(54) Title: CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN INTERNAL RIBOSOME ENTRY SITE

(57) Abstract: Disclosed herein are replication-competent adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site. Methods for the preparation and use of such vectors are also provided. The vectors provide target cell-specific virus replication in applications such as cancer therapy and gene therapy.

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CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN INTERNAL RIBOSOME ENTRY SITE

TECHNICAL FIELD

5   This invention relates to new replication competent adenovirus vectors comprising an internal ribosome entry site which replicate preferentially in target cells. The present invention also relates to cell transduction using adenovirus vectors comprising an internal ribosome entry site.

BACKGROUND

10   Diseases involving altered cell proliferation, particularly hyperproliferation, constitute an important health problem. For example, despite numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes

15   of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Neoplasia resulting in benign tumors can usually be completely cured by surgical removal of the tumor mass. If a tumor becomes malignant, as manifested by invasion of surrounding tissue, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much less likely to

20   be eradicated.

        Excluding basal cell carcinoma, there are over one million new cases of cancer per year in the United States alone, and cancer accounts for over one half million deaths per year in this country. In the world as a whole, the five most common cancers are those of lung, stomach, breast, colon/rectum, and uterine cervix, and the total number of new cases

25   per year is over 6 million.

        In the United States, transitional cell carcinoma (TCC) accounts for 90 to 95 percent of all tumors of the bladder. Squamous cell carcinoma (SCC) represents 5 to 10 percent, and adenocarcinoma approximately 1 to 2 percent. Squamous cell and adenomatous elements are often found in association with transitional cell tumors, especially with high

30   grade tumors. Bladder cancer is generally divided into superficial and invasive disease. A critical factor is the distinction between those tumors that are confined to the mucosa and those that have penetrated the basement membrane and extended into the lamina propria.

The term "superficial bladder tumor" is generally used to represent a tumor that has not invaded the muscularis. Invasive tumors are described as those that have invaded the muscularis propria, the perivesical fibroadipose tissue, or adjacent structures. Carcinoma in situ (CIS) is a high grade and aggressive manifestation of TCC of the bladder that has a highly variable course.

A number of urothelial cell-specific proteins have been described, among which are the uroplakins. Uroplakins (UP), including UPIa and UPIb (27 and 28 kDa, respectively), UPII (15 kDa), and UPIII (47 kDa), are members of a group of integral membrane proteins that are major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian urothelium and may play a role as a permeability barrier and/or as a physical stabilizer of the urothelial apical surface. Wu et al. (1994) *J. Biol. Chem.* 269:13716-13724. UPs are bladder-specific proteins, and are expressed on a significant proportion of urothelial-derived tumors, including about 88% of transitional cell carcinomas. Moll et al. (1995) *Am. J. Pathol.* 147:1383-1397; and Wu et al. (1998) *Cancer Res.* 58:1291-1297. The control of the expression of the human UPII has been studied, and a 3.6-kb region upstream of the mouse UPII gene has been identified which can confer urothelial-specific transcription on heterologous genes (Lin et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:679-683). See also, U.S. Patent Nos. 5,824,543 and 6,001,646.

Melanoma, a malignant neoplasm derived from melanocytes of the skin and other sites, has been increasing in incidence worldwide. The American Joint Committee on Cancer recognizes five different forms of extraocular melanoma occurring in humans: lentigo maligna melanoma; radial spreading; nodular; acral lentiginous; and unclassified. Known melanoma-associated antigens can be classified into three main groups: tumor-associated testis-specific antigens MAGE, BAGE, GAGE, and PRAME; melanocyte differentiation antigens tyrosinase, Melan-A/MART-1 (for Melanoma Antigen Recognized by T cells), gp100, tyrosinase related protein-1(TRP-1), tyrosinase related protein-2 (TRP-2); and mutated or aberrantly expressed antigens MUM-1, cyclin-dependent kinase 4 (CDK4), beta-catenin, gp100-in4, p15, and N-acetylglucosaminyltransferase V. See, for example, Kirkin et al. (1998) *Exp. Clin. Immunogenet.* 15:19-32. Tyrosinase, TRP-1, and TRP-2 are enzymes involved in melanin biosynthesis and are specifically expressed in melanocytes. Antigenic epitopes of MART-1 have been studied extensively, with the aim of developing a melanoma vaccine. An immunodominant epitope, MART-1(27-35) has been reported to be recognized by a majority of CD8+ cytotoxic T cell clones generated to

MART-1. These MART-1(27-35)-specific CTLs specifically lyse autologous tumor cell lines expressing the epitope. Faure and Kourilsky (1998) *Crit. Rev. Immunol.* 18:77-86. However, others have reported that presence of such CTLs is not accompanied by a significant clinical response. Rivoltini et al. (1998) *Crit. Rev. Immunol.* 18:55-63.

5   A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells without affecting the functions of normal cells. For example, in traditional chemotherapy of prostate cancer, the therapeutic ratio, (*i.e.*, the ratio of tumor cell killing to normal cell killing) is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and

10   prophylaxis of neoplasia are needed.

        Accordingly, the development of more specific, targeted forms of cancer therapy, especially for cancers that are difficult to treat successfully, is of particular interest. In contrast to conventional cancer therapies, which result in relatively non-specific and often serious toxicity, more specific treatment modalities, which inhibit or kill malignant cells

15   selectively while leaving healthy cells intact, are required.

        Gene therapy, whereby a gene of interest is introduced into a malignant cell, has been attempted as an approach to treatment of many cancers. See, for example, Boulikas (1997) *Anticancer Res.* 17:1471-1505, for a description of gene therapy for prostate cancer. A gene of interest can encode a protein which is converted into a toxic substance upon

20   treatment with another compound, or it can encode an enzyme that converts a prodrug to a drug. For example, introduction of the herpes simplex virus gene encoding thymidine kinase (HSV-tk) renders cells conditionally sensitive to ganciclovir. Zjilstra *et al.* (1989) *Nature* 342: 435; Mansour *et al.* (1988) *Nature* 336: 348; Johnson *et al.* (1989) *Science* 245: 1234; Adair *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 4574; Capecchi (1989)

25   *Science* 244: 1288. Alternatively, a gene of interest can encode a compound that is directly toxic, such as, for example, diphtheria toxin. To render these treatments specific to cancer cells, the gene of interest is placed under control of a transcriptional regulatory element (TRE) that is specifically (*i.e.*, preferentially) active in the cancer cells. Cell- or tissue-specific expression can be achieved by using a TRE with cell-specific enhancers and/or

30   promoters. See generally Huber *et al.* (1995) *Adv. Drug Delivery Reviews* 17:279-292.

        A number of viral vectors and non-viral delivery systems (*e.g.*, liposomes), have been developed for gene transfer. Of the viruses proposed for gene transfer, adenoviruses are among the most easily produced and purified. Adenovirus also has the advantage of a

high efficiency of transduction (*i.e.*, introduction of the gene of interest into the target cell)
and does not require cell proliferation for efficient transduction. In addition, adenovirus
can infect a wide variety of cells *in vitro* and *in vivo*. For general background references
regarding adenovirus and development of adenoviral vector systems, see Graham *et al.*

5   (1973) *Virology* 52:456-467; Takiff *et al.* (1981) *Lancet* 11:832-834; Berkner *et al.* (1983)
Nucleic Acid Research 11: 6003-6020; Graham (1984) *EMBO J* 3:2917-2922; Bett *et al.*
(1993) *J. Virology* 67:5911-5921; and Bett *et al.* (1994) *Proc. Natl. Acad. Sci. USA*
91:8802-8806.

Adenoviruses generally undergo a lytic replication cycle following infection of a

10   host cell. In addition to lysing the infected cell, the replicative process of adenovirus
blocks the transport and translation host cell mRNA, thus inhibiting cellular protein
synthesis. For a review of adenoviruses and adenovirus replication, see Shenk, T. and
Horwitz, M.S., *Virology*, third edition, Fields, B.N. *et al.*, eds., Raven Press Limited, New
York (1996), Chapters 67 and 68, respectively.

15   When used for gene transfer, adenovirus vectors are often designed to be
replication-defective and are thus deliberately engineered to fail to replicate in the target
cell. In these vectors, the early adenovirus gene products E1A and/or E1B are often
deleted, and the gene to be transduced is commonly inserted into the E1A and/or E1B
region of the deleted virus genome. Bett *et al.* (1994) *supra.* Such vectors are propagated

20   in packaging cell lines such as the 293 line, which provides E1A and E1B functions in
trans. Graham *et al.* (1987) *J. Gen. Virol* 36:59-72; Graham (1977) *J. Gen. Virol.* 68:937-
940. The use of replication-defective adenovirus vectors as vehicles for efficient
transduction of genes has been described by, *inter alia*, Stratford-Perricaudet (1990)
Human Gene Therapy 1:241-256; Rosenfeld (1991) *Science* 252:431-434; Wang *et al.*

25   (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe *et al.* (1992) *Nature Gen.* 1:372-378;
Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld *et al.* (1992)
Cell 68:143-155; Stratford-Perricaudet *et al.* (1992) *J. Clin. Invest.* 90:626-630; Le Gal
Le Salle *et al.* (1993) *Science* 259:988-990; Mastrangeli *et al.* (1993) *J. Clin. Invest.*
91:225-234; Ragot *et al.* (1993) *Nature* 361:647-650; Hayaski *et al.* (1994) *J. Biol. Chem.*

30   269:23872-23875; and Bett *et al.* (1994) *supra.*

In the treatment of cancer by replication-defective adenoviruses, the host immune
response limits the duration of repeat doses at two levels. First, the capsid proteins of the
adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently

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expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert a prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle, coupled with the

5   transient nature of gene expression. Despite these limitations, development of adenoviral vectors for gene therapy has focused almost exclusively on the use of the virus as a vehicle for introducing a gene of interest, not as an effector in itself. In fact, replication of adenovirus vectors has been viewed as an undesirable result, largely due to the host immune response.

10   More recently, however, the use of adenovirus vectors as effectors has been described. International Patent Application Nos. PCT/US98/04080, PCT/US98/04084, PCT/US98/04133, PCT/US98/04132, PCT/US98/16312, PCT/US95/00845, PCT/US96/10838, PCT/EP98/07380 and U.S. Pat. No. 5,998,205. Adenovirus E1A and E1B genes are disclosed in Rao et al. (1992, *Proc. Natl. Acad. Sci.* USA vol. 89: 7742-

15   7746).

Replication-competent adenovirus vectors, which take advantage of the cytotoxic effects associated with adenovirus replication, have recently been described as agents for effecting selective cell growth inhibition. In such systems, a cell-specific transcriptional regulatory element (TRE) is used to control the expression of a gene essential for viral

20   replication, thus limiting viral replication to cells in which the TRE is functional. *See,* for example International Patent Application No. PCT/EP99/07380, Henderson *et al.,* U.S. Patent No. 5,698,443; Hallenbeck *et al.,* PCT/US95/15455 and U.S. Patent No. 5,998,205; Rodriguez *et al.* (1997) *Cancer Res.* 57:2559-2563.

PCT publication PCT/US98/04080 discloses replication-competent, target cell-

25   specific adenovirus vectors comprising heterologous TREs, such as those regulating expression of prostate-specific antigen (PSA), probasin (PB), α-fetoprotein (AFP), kallikrien (*hKLK2*), mucin (*MUC1*) and carcinoembryonic antigen (CEA). PCT/US98/04084 discloses replication-competent adenovirus vectors comprising an α-fetoprotein (AFP) TRE that replicate specifically in cells expressing AFP, such as

30   hepatoma cells.

Internal ribosome entry sites (IRES) are sequences which initiate translation from an internal initiation codon (usually AUG) within a bi-or multi- cistronic RNA transcript

continuing multiple protein coding regions. IRES have been characterized in
encephalomyocarditis virus and related picornaviruses. *See,* for example, Jackson *et al.*
(1995) *RNA* 1: 985-1000 and Herman (1989) *Trends in Biochemical Sciences* 14(6): 219-
222. IRES sequences are also detected in mRNAs from other viruses such as cardiovirus,

5   rhinovirus, aphthovirus, hepatitis C virus (HCV), Friend murine leukemia virus (FrMLV)
and Moloney murine leukemia virus (MoMLV). The presence of IRES in cellular RNAs
has also been described. Examples of cellular mRNAs containing IRES include those
encoding immunoglobulin heavy-chain binding protein (BiP), vascular endothelial growth
factor (VEGF), fibroblast growth factor 2, insulin-like growth factor, translational initiation

10   factor eIF4G, and the yeast transcription factors TFIID and HAP4. *See,* for example, ;
Macejak *et al.* (1991) *Nature* 353:90-94; Oh *et al.* (1992) *Genes Dev.* 6:1643-1653;
Vagner *et al.* (1995) *Mol. Cell. Biol.* 15:35-44; He *et al.* (1996) *Proc. Natl. Acad. Sci USA*
93:7274-7278; He *et al.* (1996) *Gene* 175:121-125; Tomanin *et al.* (1997) *Gene* 193:129-
140; Gambotto *et al.* (1999) *Cancer Gene Therapy* 6:45-53; Qiao *et al.* (1999) *Cancer*

15   *Gene Therapy* 6:373-379. Expression vectors containing IRES elements have been
described. *See,* for example, International Patent Application No. PCT/US98/03699 and
International Patent Application No. PCT/EP98/07380.

        Thus, there is a continuing need for improved replication-competent adenovirus
vectors in which cell-specific replication can be further enhanced, while minimizing the

20   extent of replication in non-target (*i.e.,* non-cancerous cells).

        The disclosure of all patents and publications cited herein are incorporated by
reference in their entirety.

                            SUMMARY OF THE INVENTION

25   The present invention provides improved replication competent adenovirus vectors
comprising co-transcribed first and second genes under transcriptional control of a
heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the
second gene is under translational control of an internal ribosome entry site (IRES). In one
embodiment, the first and second genes are co-transcribed as a single mRNA and the

30   second gene has a mutation in or deletion of its endogenous promoter. The present
invention further provides host cells and methods using the adenovirus vectors.

        In one aspect, the first and/or second genes are adenovirus genes and in another
aspect, the first and/or second adenovirus genes are essential for viral replication. An

essential gene can be an early viral gene, including for example, E1A; E1B; E2; and/or E4, or a late viral gene. In another aspect an early gene is E3.

In one embodiment, the first gene is an adenovirus gene and the second gene is a therapeutic gene. In another embodiment, both genes are adenovirus genes. In an
5   additional embodiment, the first adenovirus gene is E1A, and the second adenovirus gene is E1B. Optionally, the endogenous promoter for one of the co-transcribed adenovirus gene essential for viral replication, such as for example, E1A, is deleted and/or mutated such that the gene is under sole transcriptional control of a target cell-specific TRE.

In another aspect, the present invention provides adenovirus vectors comprising an
10   adenovirus gene essential for viral replication under control of a target cell-specific TRE, wherein said adenovirus gene has a mutation of or deletion in its endogenous promoter. In one embodiment, the adenovirus gene is essential for viral replication. In another embodiment, the adenovirus gene is E1A wherein the E1A promoter is deleted and wherein the E1A gene is under transcriptional control of a heterologous cell-specific TRE. In
15   another embodiment, the adenovirus gene is E1B wherein the E1B promoter is deleted and wherein the E1B gene is under transcriptional control of a heterologous cell-specific TRE.

In another aspect, the present invention provides adenovirus vectors comprising E1B under control of a target cell-specific TRE, wherein said E1B has a deletion in or mutation of the 19-kDa region of E1B, that encodes a product shown to inhibit apoptosis.
20   In other embodiments, an enhancer element for the first and/or second adenovirus genes is inactivated. The present invention provides an adenovirus vector comprising E1A wherein an E1A enhancer is inactivated. In yet other embodiments, the present invention provides an adenovirus vector comprising E1A wherein the E1A promoter is inactivated and E1A enhancer I is inactivated. In further embodiments, the present invention provides
25   an adenovirus vector comprising a TRE which has its endogenous silencer element inactivated.

Any TRE which directs cell-specific expression can be used in the disclosed vectors. In one embodiment, TREs include, for example, TREs specific for prostate cancer cells, breast cancer cells, hepatoma cells, melanoma cells, bladder cells and/or colon cancer
30   cells. In another embodiment, the TREs include, probasin (PB) TRE; prostate-specific antigen (PSA) TRE; mucin (*MUC1*) TRE; α-fetoprotein (AFP) TRE; *hKLK2* TRE; tyrosinase TRE; human uroplakin II TRE (hUPII) and carcinoembryonic antigen (CEA)

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TRE. In other embodiments, the target cell-specific TRE is a cell status-specific TRE. In yet other embodiments, the target cell-specific TRE is a tissue specific TRE.

In additional embodiments, the adenovirus vector comprises at least one additional co-transcribed gene under the control of the cell-specific TRE. In another embodiment, an additional co-transcribed gene is under the translational control of an IRES.

In another aspect of the present invention, adenovirus vectors further comprise a transgene such as, for example, a cytotoxic gene. In one embodiment, the transgene is under the transcriptional control of the same TRE as the first gene and second genes and optionally under the translational control of an internal ribosome entry site. In another embodiment, the transgene is under the transcriptional control of a different TRE that is functional in the same cell as the TRE regulating transcription of the first and second genes and optionally under the translational control of an IRES.

The present invention also provides compositions comprising the replication-competent adenovirus vectors described herein. In one embodiment, the compositions further comprise a pharmaceutically acceptable excipient. The present invention also provides kits comprising the replication-competent adnenovirus vectors described herein.

Host cells comprising the disclosed adenovirus vectors are also provided. Host cells include those used for propagation of a vector and those into which a vector is introduced for therapeutic purposes.

In another aspect, methods are provided for propagating replication-competent adenovirus vectors of the present invention specific for mammalian cells which permit the function of a target cell-specific TRE, said method comprising combining an adenovirus vector(s) described herein with mammalian cells that permit the function of a target cell-specific TRE, such that the adenovirus vector(s) enters the cell, whereby said adenovirus is propagated.

In another aspect, methods are provided for conferring selective cytotoxicity in target cells, comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, more particularly a target tumor cell, comprising contacting a tumor cell with an adenovirus vector(s) of the invention such that the adenovirus vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

In another aspect, methods are provided for detecting a cell which allows the function of a target cell-specific TRE, which comprise contacting a cell in a biological sample with an adenovirus vector(s) of the invention, and detecting replication of the adenovirus vector(s), if any.

5   In another aspect, methods are provided for modifying the genotype of a target cell, comprising contacting the cell with an adenovirus vector as described herein, wherein the adenovirus vector enters the cell.

The present invention provides an adenovirus vector comprising an adenovirus gene, wherein said adenovirus gene is under transcriptional control of a melanocyte-
10   specific TRE. In another embodiment, a melanocyte-specific TRE is human. In another embodiment, a melanocyte-specific TRE comprises a melanocyte-specific promoter and a heterologous enhancer. In other embodiments, a melanocyte-specific TRE comprises a melanocyte-specific promoter. In other embodiments, a melanocyte-specific TRE comprises a melanocyte-specific enhancer and a heterologous promoter. In other
15   embodiments, a melanocyte-specific TRE comprises a melanocyte-specific promoter and a melanocyte-specific enhancer.

In some embodiments, the adenovirus gene under transcriptional control of a melanocyte-specific TRE is an adenovirus gene essential for replication. In some embodiments, the adenoviral gene essential for replication is an early gene. In another
20   embodiment, the early gene is E1A. In another embodiment, the early gene is E1B. In yet another embodiment, both E1A and E1B are under transcriptional control of a melanocyte-specific TRE. In further embodiments, the adenovirus gene essential for replication is E1B, and E1B has a deletion in the 19-kDa region.

In some embodiments, the melanocyte-specific TRE is derived from the 5' flanking
25   region of a tyrosinase gene. In other embodiments, the melanocyte-specific TRE is derived from the 5' flanking region of a tyrosinase related protein-1 gene. In other embodiments, the melanocyte-specific TRE is derived from the 5'-flanking region of a tyrosinase related protein-2 gene. In other embodiments, the melanocyte-specific TRE is derived from the 5' flanking region of a MART-1 gene. In other embodiments, the melanocyte-specific TRE is
30   derived from the 5'-flanking region of a gene which is aberrantly expressed in melanomas.

In other embodiments, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a melanocyte-specific TRE; and (b) an

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E3 region. In some of these embodiments the E3 region is under transcriptional control of a melanocyte-specific TRE.

In another aspect, the invention provides a host cell comprising the melanocyte specific adenovirus vector(s) described herein.

5   In another aspect, the invention provides pharmaceutical compositions comprising a melanocyte specific adenovirus vector(s) described herein.

In another aspect, the invention provides kits which contain a melanocyte adenoviral vector(s) described herein.

In another aspect, methods are provided for conferring selective cytoxicity in target

10   cells (i.e., cells which permit or induce a melanocyte-specific TRE to function), comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

In another aspect, methods are provided for propagating an adenovirus specific for melanocytes, said method comprising combining an melanocyte specific adenovirus

15   vector(s) described herein with melanocytes, whereby said adenovirus is propagated.

The invention further provides methods of suppressing melanoma cell growth, comprising contacting a melanoma cell with a melanocyte specific adenoviral vector of the invention such that the adenoviral vector enters the melanoma cell and exhibits selective cytotoxicity for the melanoma cell.

20   In another aspect, methods are provided for detecting melanocytes, including melanoma cells, in a biological sample, comprising contacting cells of a biological sample with a melanocyte adenovirus vector(s) described herein, and detecting replication of the adenovirus vector, if any.

25   BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of plasmid construct CP627 as described in Example 1.

Figures 2A-2B is a series of schematic depictions of various adenoviruses described herein.

Figure 3 depicts the replication efficiency of different viruses as described in

30   Example 4.

Figures 4A and 4B show viral yield for different liver-specific vectors in different cell types.

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Figure 5 is a schematic representation of adenovirus vectors comprising AFP-TRE with and without IRES.

Figure 6 depicts an E3 region.

Figure 7 is a schematic representation of adenovirus vectors described herein.

Figure 8 depicts in vivo antitumor activity of CV890 containing an IRES. This figure depicts the results of a HepG2 Xenograph treated with CV790 or CV890.

Figure 9 depicts an ADP nucleotide and amino acid sequence.

Figure 10 depicts an IC_{50} isobologram of doxorubicin and CV 890 on Hep3B cells at day 5.

Figure 11 depicts in vivo efficacy of CV890 with doxorubicin. Hep3B nude mouse xenografts were grouped (n=6) and treated with CV890 alone ($1x10^{11}$ particles/dose, iv), doxorubicin alone (10mg/kg, ip), CV890 and doxorubicin combination ($1x10^{11}$ particles of CV890 through tail vein and 10mg/kg doxorubicin ip), or vehicle control. Tumor size was measured weekly and the tumor volume were normalized as 100% at the day of treatment. Error bars represent the standard error of the mean.

Figure 12 shows the virus yield of CV802, CV882 and CV884 in cell lines.

MODES FOR CARRYING OUT THE INVENTION

We have discovered and constructed improved adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site (IRES). In one embodiment, the first and second genes are co-transcribed as a single mRNA and the second gene has a mutation in or deletion of its endogenous promoter. In another embodiment, at least one of the genes is an adenovirus gene and in yet another embodiment, both genes are adenovirus genes, including adenovirus genes that are essential for viral replication. The adenovirus vector may comprise a gene that contributes to cytotoxicity (whether direct and/or indirect), and/or causes cell death. An example of an adenovirus gene that contributes to cytotoxicity includes, but is not limited to, the adenovirus death protein gene.

In some aspects of the present invention, an adenovirus vector comprising co-transcribed first and second genes under transcriptional control of a target cell-specific TRE, wherein the second gene is under translational control of an IRES, exhibits greater specificity for the target cell than an adenovirus vector comprising a target cell-specific

TRE operably linked to a gene and lacking an IRES. In some embodiments, specificity is conferred by preferential transcription and/or translation of the first and second genes due to the presence of a target cell specific TRE. In other embodiments, specificity is conferred by preferential replication of the adenovirus vectors in target cells due to the target cell-

5   specific TRE driving transcription of a gene essential for replication.

Also disclosed herein are IRES containing adenovirus vectors comprising an adenovirus gene essential for viral replication wherein said essential gene has a mutation in or deletion of its endogenous promoter. In an embodiment disclosed herein, the adenovirus vectors comprise the adenovirus early gene E1A which has a deletion of its endogenous

10   promoter. In another embodiment disclosed herein, the adenovirus vectors comprise the adenovirus early gene E1B which has a deletion of its endogenous promoter. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

In another aspect, the adenovirus vectors disclosed herein comprise an adenovirus gene essential for viral replication wherein said essential gene has a mutation in or deletion

15   of its endogenous enhancer. In one embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter. In one embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in E1A enhancer 1. In a further embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its

20   endogenous promoter and a mutation of or deletion in the E1A enhancer. In an additional embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter and the adenovirus early gene E1B which has a mutation of or deletion in its endogenous promoter. In an additional embodiment, the adenovirus vector comprises the adenovirus early gene E1A, which has a

25   mutation of or deletion in its endogenous promoter and a mutation of or deletion in the E1A enhancer I, and the adenovirus early gene E1B which has a mutation of or deletion in its endogenous promoter. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

The replication-competent adenovirus vectors of the present invention take

30   advantage of what has been heretofore considered an undesirable aspect of adenovirus vectors, namely their replication and possible concomitant immunogenicity. Runaway infection is prevented due to the cell-specific requirements for viral replication. Without wishing to be bound by any particular theory, it is noted that production of adenovirus

proteins can serve to activate and/or stimulate the immune system, either generally or specifically, toward target cells producing adenoviral proteins. This type of immune stimulation can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

5   The adenovirus vectors of the present invention comprising an intergenic IRES element(s) which links the translation of two or more genes, reflects an improvement over vector constructs which use identical control regions to drive expression of two or more desired genes in that any potential for homologous recombination based on the presence of homologous control regions in the vector is removed. As demonstrated herein, adenovirus 10   vectors comprising an IRES are stable and in some embodiments provide better specificity than vectors not containing an IRES. Another advantage of an adenovirus vector comprising an intergenic IRES is that the use of an IRES rather than a second TRE may provide additional space in the vector for an additional gene(s) such as a therapeutic gene.

          Thus, the adenovirus vectors comprising a second gene under control of an IRES 15   retain a high level of target cell specificity and remain stable in the target cell. Accordingly, in one aspect of the invention, the viral vectors disclosed herein comprise at least one IRES within a multicistronic transcript, wherein production of the multicistronic transcript is regulated by a heterologous, target cell-specific TRE. For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous 20   promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter does not interfere with transcription of the second gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred that the initiation codon of the second gene is removed and that the IRES and the second gene are 25   in frame. Alternatively, if the IRES does not contain an initiation codon or if the initiation codon is removed from the IRES, the initiation codon of the second gene is used. In one embodiment, the adenovirus vectors comprises the adenovirus essential genes, E1A and E1B genes, under the transcriptional control of a heterologous, cell-specific TRE, and an IRES introduced between E1A and E1B. Thus, both E1A and E1B are under common 30   transcriptional control, and translation of E1B coding region is obtained by virtue of the presence of the IRES. In one embodiment, E1A has its endogenous promoter deleted. In another embodiment, E1A has an endogenous enhancer deleted and in yet an additional embodiment, E1A has its endogenous promoter deleted and E1A enhancer I deleted. In

another embodiment, E1B has its endogenous promoter deleted. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

To provide cytotoxicity to target cells, one or more transgenes having a cytotoxic effect may be present in the vector. Additionally, or alternatively, an adenovirus gene that

5   contributes to cytotoxicity and/or cell death, such as the adenovirus death protein (ADP) gene, can be included in the vector, optionally under the selective transcriptional control of a heterologous TRE and optionally under the translational control of an IRES.

Examples of target cells include neoplastic cells, although any cell for which it is desirable and/or tolerable to sustain a cytotoxic activity can be a target cell. By combining

10   an adenovirus vector(s) comprising a target cell-specific TRE with a mixture of target and non-target cells, *in vitro* or *in vivo*, the vector(s) preferentially replicates in the target cells, causing cytotoxic and/or cytolytic effects. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic activity, replication of the vector(s) is significantly reduced, lessening the probability of runaway infection and undesirable bystander effects.

15   *In vitro* cultures can be retained to continually monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for the presence of the undesirable target cell, *e.g.*, a cancer cell in which the target cell-specific TRE is functional. The adenovirus vectors of the present invention can also be used in *ex vivo* procedures wherein desirable biological samples comprising target cells are removed from the animal, subjected to

20   exposure to an adenovirus vector of the present invention comprising a target cell-specific TRE and then replaced within the animal.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques),

25   microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook *et al.*, 1989); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Wei &

30   C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987 and annual updates); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991 and annual updates).

For techniques related to adenovirus, see, *inter alia*, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

5   **Definitions**

As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83)

10   and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000). The present invention encompasses the use of any IRES element which is able to promote direct internal ribosome entry to the initiation codon of a cistron. "Under translational control of an IRES" as used herein means that translation is associated with the IRES and proceeds in a cap-independent manner. Examples of "IRES" known in the art include, but are not limited

15   to IRES obtainable from picornavirus (Jackson et al., 1990, *Trends Biochem Sci* 15(12):477-483); and IRES obtainable from viral or cellular mRNA sources, such as for example, immunogloublin heavy-chain binding protein (BiP), the vascular endothelial growth factor (VEGF) (Huez et al. (1998) *Mol. Cell. Biol.* 18(11):6178-6190), the fibroblast growth factor 2, and insulin-like growth factor, the translational initiation factor

20   eIF4G, yeast transcription factors TFIID and HAP4. IRES have also been reported in different viruses such as cardiovirus, rhinovirus, aphthovirus, HCV, Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV). As used herein, "IRES" encompasses functional variations of IRES sequences as long as the variation is able to promote direct internal ribosome entry to the initiation codon of a cistron. In

25   preferred embodiments, the IRES is mammalian. In other embodiments, the IRES is viral or protozoan. In one illustrative embodiment disclosed herein, the IRES is obtainable from encephelomycarditis virus (ECMV) (commercially available from Novogen, Duke et al. (1992) *J. Virol* 66(3):1602-1609). In another illustrative embodiment disclosed herein, the IRES is from VEGF. Table I and Table II disclose a variety of IRES sequences useful in

30   the present invention.

A "multicistronic transcript" refers to an mRNA molecule which contains more than one protein coding region, or cistron. A mRNA comprising two coding regions is denoted a "bicistronic transcript." The "5'-proximal" coding region or cistron is the coding

region whose translation initiation codon (usually AUG) is closest to the 5'-end of a
multicistronic mRNA molecule. A "5'-distal" coding region or cistron is one whose
translation initiation codon (usually AUG) is not the closest initiation codon to the 5' end
of the mRNA. The terms "5'-distal" and "downstream" are used synonymously to refer to

5   coding regions that are not adjacent to the 5' end of a mRNA molecule.

As used herein, "co-transcribed" means that two (or more) coding regions of
polynucleotides are under transcriptional control of single transcriptional control element.

A "gene" refers to a coding region of a polynucleotide. A "gene" may or may not
include non-coding sequences and/or regulatory elements.

10   As used herein, a "transcription response element" or "transcriptional regulatory
element", or "TRE" is a polynucleotide sequence, preferably a DNA sequence, which
increases transcription of an operably linked polynucleotide sequence in a host cell that
allows that TRE to function. A TRE can comprise an enhancer and/or a promoter. A
"transcriptional regulatory sequence" is a TRE. A "target cell-specific transcriptional

15   response element" or "target cell-specific TRE" is a polynucleotide sequence, preferably a
DNA sequence, which is preferentially functional in a specific type of cell, that is, a target
cell. Accordingly, a target cell-specific TRE transcribes an operably linked polynucleotide
sequence in a target cell that allows the target cell-specific TRE to function. The term
"target cell-specific", as used herein, is intended to include cell type specificity, tissue

20   specificity, developmental stage specificity, and tumor specificity, as well as specificity for
a cancerous state of a given target cell. "Target cell-specific TRE" includes cell type-
specific and cell status-specific TRE, as well as "composite" TREs. The term "composite
TRE" includes a TRE which comprises both a cell type-specific and a cell status-specific
TRE. A target cell-specific TRE can also include a heterologous component, including, for

25   example, an SV40 or a cytomegalovirus (CMV) promoter(s). An example of a target cell
specific TRE which is tissue specific is a CMV TRE which contains both promoter(s) and
enhancer(s).

As described in more detail herein, a target cell-specific TRE can comprise any
number of configurations, including, but not limited to, a target cell-specific promoter; and

30   target cell-specific enhancer; a heterologous promoter and a target cell-specific enhancer; a
target cell-specific promoter and a heterologous enhancer; a heterologous promoter and a
heterologous enhancer; and multimers of the foregoing. The promoter and enhancer
components of a target cell-specific TRE may be in any orientation and/or distance from

the coding sequence of interest, as long as the desired target cell-specific transcriptional

activity is obtained. Transcriptional activation can be measured in a number of ways

known in the art (and described in more detail below), but is generally measured by

detection and/or quantitation of mRNA or the protein product of the coding sequence under

5   control of (i.e., operably linked to) the target cell-specific TRE. As discussed herein, a

target cell-specific TRE can be of varying lengths, and of varying sequence composition.

As used herein, the term "cell status-specific TRE" is preferentially functional, i.e., confers

transcriptional activation on an operably linked polynucleotide in a cell which allows a cell

status-specific TRE to function, i.e., a cell which exhibits a particular physiological

10   condition, including, but not limited to, an aberrant physiological state. "Cell status" thus

refers to a given, or particular, physiological state (or condition) of a cell, which is

reversible and/or progressive. The physiological state may be generated internally or

externally; for example, it may be a metabolic state (such as in response to conditions of

low oxygen), or it may be generated due to heat or ionizing radiation. "Cell status" is

15   distinct from a "cell type", which relates to a differentiation state of a cell, which under

normal conditions is irreversible. Generally (but not necessarily), as discussed herein, a

cell status is embodied in an aberrant physiological state, examples of which are given

below.

A "functional portion" of a target cell-specific TRE is one which confers target cell-

20   specific transcription on an operably linked gene or coding region, such that the operably

linked gene or coding region is preferentially expressed in the target cells.

By "transcriptional activation" or an "increase in transcription," it is intended that

transcription is increased above basal levels in the target cell (i.e., target cell) by at least

about 2 fold, preferably at least about 5 fold, preferably at least about 10 fold, more

25   preferably at least about 20 fold, more preferably at least about 50 fold, more preferably at

least about 100 fold, more preferably at least about 200 fold, even more preferably at least

about 400 fold to about 500 fold, even more preferably at least about 1000 fold. Basal

levels are generally the level of activity (if any) in a non-target cell (i.e., a different cell

type), or the level of activity (if any) of a reporter construct lacking a target cell-specific

30   TRE as tested in a target cell line.

A "functionally-preserved variant" of a target cell-specific TRE is a target cell-

specific TRE which differs from another target cell-specific TRE, but still retains target

cell-specific transcription activity, although the degree of activation may be altered (as

discussed below). The difference in a target cell-specific TRE can be due to differences in linear sequence, arising from, for example, single base mutation(s), addition(s), deletion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a target cell-specific TRE. For example,

5   certain point mutations within sequences of TREs have been shown to decrease transcription factor binding and stimulation of transcription. See Blackwood, et al. (1998) *Science* 281:60-63 and Smith et al. (1997) *J. Biol. Chem.* 272:27493-27496. One of skill in the art would recognize that some alterations of bases in and around transcription factor binding sites are more likely to negatively affect stimulation of transcription and cell-

10   specificity, while alterations in bases which are not involved in transcription factor binding are not as likely to have such effects. Certain mutations are also capable of increasing TRE activity. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in TRE functional and TRE non-functional cells. Additionally, one of

15   skill in the art would recognize that point mutations and deletions can be made to a TRE sequence without altering the ability of the sequence to regulate transcription.

As used herein, a TRE derived from a specific gene is referred to by the gene from which it was derived and is a polynucleotide sequence which regulates transcription of an operably linked polynucleotide sequence in a host cell that expresses said gene. For

20   example, as used herein, a "human glandular kallikrein transcriptional regulatory element", or "*hKLK2*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows an *hKLK2*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor, such as a prostate cell. An

25   *hKLK2*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of an *hKLK2* promoter and/or an *hKLK2* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "probasin (*PB*) transcriptional regulatory element", or "*PB*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases

30   transcription of an operably-linked polynucleotide sequence in a host cell that allows a *PB*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PB*-TRE is

thus responsive to the binding of androgen receptor and comprises at least a portion of a *PB* promoter and/or a *PB* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "prostate-specific antigen (*PSA*) transcriptional regulatory element", or "*PSA*-TRE", or "*PSE*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *PSA*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PSA*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PSA* promoter and/or a *PSA* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "carcinoembryonic antigen (*CEA*) transcriptional regulatory element", or "*CEA*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *CEA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses CEA. The *CEA*-TRE is responsive to transcription factors and/or co-factor(s) associated with CEA-producing cells and comprises at least a portion of the *CEA* promoter and/or enhancer.

As used herein, an "α-fetoprotein (*AFP*) transcriptional regulatory element", or "*AFP*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably linked polynucleotide sequence) in a host cell that allows an *AFP*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses AFP. The *AFP*-TRE is responsive to transcription factors and/or co-factor(s) associated with AFP-producing cells and comprises at least a portion of the *AFP* promoter and/or enhancer.

As used herein, an "a mucin gene (*MUC*) transcriptional regulatory element", or "*MUC1*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably-linked polynucleotide sequence) in a host cell that allows a *MUC1*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses MUC1. The *MUC1*-TRE is responsive to transcription factors and/or co-factor(s) associated with MUC1-producing cells and comprises at least a portion of the *MUC1* promoter and/or enhancer.

As used herein, a "urothelial cell-specific transcriptional response element", or "urothelial cell-specific TRE" is polynucleotide sequence, preferably a DNA sequence,

which increases transcription of an operably linked polynucleotide sequence in a host cell that allows a urothelial-specific TRE to function, i.e., a target cell. A variety of urothelial cell-specific TREs are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with urothelial cells, and comprise at least a portion of a

5   urothelial-specific promoter and/or a urothelial-specific enhancer. Methods are described herein for measuring the activity of a urothelial cell-specific TRE and thus for determining whether a given cell allows a urothelial cell-specific TRE to function.

        As used herein, a "melanocyte cell-specific transcriptional response element", or "melanocyte cell-specific TRE" is polynucleotide sequence, preferably a DNA sequence,

10   which increases transcription of an operably linked polynucleotide sequence in a host cell that allows a melanocyte-specific TRE to function, i.e., a target cell. A variety of melanocyte cell-specific TREs are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with melanocyte cells, and comprise at least a portion of a melanocyte-specific promoter and/or a melanocyte-specific enhancer.

15   Methods are described herein for measuring the activity of a melanocyte cell-specific TRE . and thus for determining whether a given cell allows a melanocyte cell-specific TRE to function.

        An "E1B 19-kDa region" (used interchangeably with "E1B 19-kDa genomic region") refers to the genomic region of the adenovirus E1B gene encoding the E1B 19-

20   kDa product. According to wild-type Ad5 , the E1B 19-kDa region is a 261bp region located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao *et al.*, *Proc. Natl. Acad. Sci. USA, 89:*7742-7746. The present invention encompasses deletion of part or all of the E1B 19-kDa region as well as embodiments wherein the E1B 19-kDa region is mutated, as long as the deletion or

25   mutation lessens or eliminates the inhibition of apoptosis associated with E1B-19kDa.

        As used herein, a target cell-specific TRE can comprise any number of configurations, including, but not limited to, a target cell-specific promoter; a target cell-specific enhancer; a target cell-specific promoter and a target cell-specific enhancer; a target cell-specific promoter and a heterologous enhancer; a heterologous promoter and a

30   target cell-specific enhancer; and multimers of the foregoing. The promoter and enhancer components of a target cell-specific TRE may be in any orientation and/or distance from the coding sequence of interest, as long as the desired target cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways

known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operably linked to) the target cell-specific TRE.

"Replicating preferentially", as used herein, means that the adenovirus replicates more in a target cell than a non-target cell. Preferably, the adenovirus replicates at a significantly higher rate in target cells than non target cells; preferably, at least about 2-fold higher, preferably, at least about 5-fold higher, more preferably, at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at least about 100-fold higher, still more preferably at least about 400- to 500-fold higher, still more preferably at least about 1000-fold higher, most preferably at least about 1×10^6 higher. Most preferably, the adenovirus replicates solely in the target cells (that is, does not replicate or replicates at a very low levels in non-target cells).

As used herein, the term "vector" refers to a polynucleotide construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors" which are designed for isolation, propagation and replication of inserted nucleotides, "expression vectors" which are designed for expression of a nucleotide sequence in a host cell, or a "viral vector" which is designed to result in the production of a recombinant virus or virus-like particle, or "shuttle vectors", which comprise the attributes of more than one type of vector.

An "adenovirus vector" or "adenoviral vector" (used interchangeably) comprises a polynucleotide construct of the invention. A polynucleotide construct of this invention may be in any of several forms, including, but not limited to, DNA, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically "mask" the molecule and/or increase half-life, and conjugated to a nonviral protein. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and

5   pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a

10   oligodeoxynucleoside phosphoramidate (P-NH2) or a mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothioate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995)

15   *Molec. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. Reference to a polynucleotide sequence (such as referring to a

20   SEQ ID NO) also includes the complement sequence.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A

25   polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included

30   in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G,

but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2.

"Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. "Operably linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

An "E3 region" (used interchangeably with "E3") is a term well understood in the art and means the region of the adenoviral genome that encodes the E3 products (discussed herein). Generally, the E3 region is located between about 28583 and 30470 of the adenoviral genome. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274.

A "portion" of the E3 region means less than the entire E3 region, and as such includes polynucleotide deletions as well as polynucleotides encoding one or more polypeptide products of the E3 region.

As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which a cell's usual biochemical or biological activities are compromised (i.e., inhibited). These activities include, but are not limited to, metabolism; cellular replication; DNA replication; transcription; translation; uptake of molecules. "Cytotoxicity" includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ^{3}H-thymidine uptake, and plaque assays.

The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows or

induces a target cell-specific TRE to function (a target cell) when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow a target cell-specific TRE to function (a non-target cell). Such cytotoxicity may be measured, for example, by plaque assays, by reduction or stabilization in size of a tumor

5   comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells, or a tissue-specific marker, e.g., a cancer marker.

In the context of adenovirus, a "heterologous polynucleotide" or "heterologous gene" or "transgene" is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell

10   prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

In the context of adenovirus, a "heterologous" promoter or enhancer is one which is not associated with or derived from an adenovirus gene.

In the context of adenovirus, an "endogenous" promoter, enhancer, or TRE is native

15   to or derived from adenovirus. In the context of promoter, an "inactivation" means that there is a mutation of or deletion in part or all of the of the endogenous promoter, ie, a modification or alteration of the endogenous promoter, such as, for example, a point mutation or insertion, which disables the function of the promoter.

In the context of a target cell-specific TRE, a "heterologous" promoter or enhancer

20   is one which is derived from a gene other than the gene from which a reference target cell-specific TRE is derived.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. Tumor cell growth can be assessed by any means known in the art,

25   including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells",

30   "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

24

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate

5   mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

"Replication" and "propagation" are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. These terms are well understood in the art. For purposes of this invention, replication involves production of

10   adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. "Replication" and "propagation" include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of

15   viral components into complete viruses; and cell lysis.

An "ADP coding sequence" is a polynucleotide that encodes ADP or a functional fragment thereof. In the context of ADP, a "functional fragment" of ADP is one that exhibits cytotoxic activity, especially cell lysis, with respect to adenoviral replication. Ways to measure cytotoxic activity are known in the art and are described herein.

20   A polynucleotide that "encodes" an ADP polypeptide is one that can be transcribed and/or translated to produce an ADP polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

An "ADP polypeptide" is a polypeptide containing at least a portion, or region, of the amino acid sequence of an ADP and which displays a function associated with ADP,

25   particularly cytotoxicity, more particularly, cell lysis. As discussed herein, these functions can be measured using techniques known in the art. It is understood that certain sequence variations may be used, due to, for example, conservative amino acid substitutions, which may provide ADP polypeptides.

"Androgen receptor," or AR, as used herein refers to a protein whose function is to

30   specifically bind to androgen and, as a consequence of the specific binding, recognize and bind to an androgen response element (ARE), following which the AR is capable of regulating transcriptional activity. The AR is a nuclear receptor that, when activated, binds to cellular androgen-responsive element(s). In normal cells the AR is activated by

androgen, but in non-normal cells (including malignant cells) the AR may be activated by non-androgenic agents, including hormones other than androgens. Encompassed in the term "androgen receptor" are mutant forms of an androgen receptor, such as those characterized by amino acid additions, insertions, truncations and deletions, as long as the

5   function is sufficiently preserved. Mutants include androgen receptors with amino acid additions, insertions, truncations and deletions, as long as the function is sufficiently preserved. In this context, a functional androgen receptor is one that binds both androgen and, upon androgen binding, an ARE.

A polynucleotide sequence that is "depicted in" a SEQ ID NO means that the

10   sequence is present as an identical contiguous sequence in the SEQ ID NO. The term encompasses portions, or regions of the SEQ ID NO as well as the entire sequence contained within the SEQ ID NO.

A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition

15   encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample"

20   encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates, and pets.

25   An "effective amount" is an amount sufficient to effect beneficial or desired results, including clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

30   A given TRE is "derived from" a given gene if it is associated with that gene in nature.

"Expression" includes transcription and/or translation.

As used herein, the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

"A," "an" and "the" include plural references unless the context clearly dictates otherwise.

5   *Internal Ribosome Entry Site (IRES)*

IRES elements were first discovered in picornavirus mRNAs (Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83) and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000). The present invention provides improved adenovirus vectors comprising co-transcribed first and second genes under transcriptional

10   control of a heterologous, target cell-specific TRE, and wherein the second gene (i.e.,
coding region) is under translational control of an internal ribosome entry site (IRES). Any IRES may be used in the adenovirus vectors of the invention, as long as they exhibit requisite function in the vectors. Example of IRES which can be used in the present invention include those provided in Table I and referenced in Table II. Examples of IRES

15   elements include the encephelomycarditis virus (EMCV) which is commercially available from Novagen (Duke et al. (1992) *J. Virol* 66(3):1602-9) the sequence for which is depicted in Table 1 (SEQ ID NO:1). Another example of an IRES element disclosed herein is the VEGF IRES (Huez et al. (1998) *Mol Cell Biol* 18(11):6178-90). This IRES has a short segment and the sequence is depicted in Table 1 (SEQ ID NO:2).

20   The IRES promotes direct internal ribosome entry to the initiation codon of a downstream cistron, leading to cap-independent translation. Thus, the product of a downstream cistron can be expressed from a bicistronic (or multicistronic) mRNA, without requiring either cleavage of a polyprotein or generation of a monocistronic mRNA. Therefore, in one illustrative embodiment of the present invention, an adenovirus vector

25   comprising E1B under translational control of an IRES allows translation of E1B from a bicistronic E1A-E1B mRNA under control of a target cell-specific TRE. Figure 7 provides a schematic representation of adenovirus constructs of the present invention.

Internal ribosome entry sites are approximately 450 nucleotides in length and are characterized by moderate conservation of primary sequence and strong conservation of

30   secondary structure. The most significant primary sequence feature of the IRES is a pyrimidine-rich site whose start is located approximately 25 nucleotides upstream of the 3' end of the IRES. *See* Jackson *et al. (1990)*.

Three major classes of picornavirus IRES have been identified and characterized:
(1) the cardio- and aphthovirus class (for example, the encephelomycarditis virus, Jang et
al. (1990) *Gene Dev* 4:1560-1572); (2) the entero- and rhinovirus class (for example,
polioviruses, Borman et al. (1994) *EMBO J.* 13:314903157); and (3)the hepatitis A virus

5   (HAV) class, Glass et al. (1993) *Virol* 193:842-852). For the first two classes, two general
principles apply. First, most of the 450-nucleotide sequence of the IRES functions to
maintain particular secondary and tertiary structures conducive to ribosome binding and
translational initiation. Second, the ribosome entry site is an AUG triplet located at the 3'
end of the IRES, approximately 25 nucleotides downstream of a conserved oligopyrimidine

10   tract. Translation initiation can occur either at the ribosome entry site (cardioviruses) or at
the next downstream AUG (entero/rhinovirus class). Initiation occurs at both sites in
aphthoviruses.

HCV and pestiviruses such as bovine viral diarrhea virus (BVDV) or classical
swine fever virus (CSFV) have 341 nt and 370 nt long 5'-UTR respectively. These 5'-UTR

15   fragments form similar RNA secondary structures and can have moderately efficient IRES
function (Tsukiyama-Kohara et al. (1992) *J. Virol.* 66:1476-1483; Frolov I et al., (1998)
RNA 4:1418-1435). Table I depicts the 5'-UTR region from HCV genome sequence
(GenBank accession D14853).

Leishmania RNA virus 1 (LRV1) is a double-stranded RNA virus. Its 128 nt long

20   5'-UTR has IRES activity to facilitate the cap-independent translation, (Maga et al. (1995)
Mol Cell Biol 15:4884-4889). This fragment also forms conserved stemloop secondary
structure and at least the front part is essential.

Recent studies showed that both Friend-murine leukemia virus (MLV) 5'-UTR and
rat retrotransposon virus-like 30S (VL30) sequences contain IRES structure of retroviral

25   origin (Torrent et al. (1996) *Hum Gene Ther* 7:603-612). These fragments are also
functional as packing signal when used in retrovirus derived vectors. Studies of avian
reticuloendotheliosis virus type A (REV-A) show that its IRES maps downstream of the
packaging/dimerization (E/DLS) sequence and the minimal IRES sequence appears to be
within a 129 nt fragment (452-580) of the 5' leader, immediately upstream of the gag AUG

30   codon (Lopez-Lastra et al. (1997) *Hum Gene Ther* 8:1855-1865).

In eukaryotic cells, translation is normally initiated by the ribosome scanning from
the capped mRNA 5' end, under the control of initiation factors. However, several cellular

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mRNAs have been found to have IRES structure to mediate the cap-independent translation (van der Velde, et al. (1999) *Int J Biochem Cell Biol.* 31:87-106). Examples are immunoglobulin heavy-chain binding protein (BiP) (Macejak et al. (1991) *Nature* 353:90-94), antennapedia mRNA of Drosophilan (Oh et al. (1992) *Gene and Dev*

5   6:1643-1653), fibroblast growth factor-2 (FGF-2) (Vagner et al. (1995) *Mol Cell Biol* 15:35-44), platelet-derived growth factor B (PDGF-B) (Bernstein et al. (1997) *J Biol Chem* 272:9356-9362), insulin-like growth factor II (Teerink et al. (1995) *Biochim Biophys Acta* 1264:403-408), and the translation initiation factor eIF4G (Gan et al. (1996) *J Biol Chem* 271:623-626). Table 1 depicts the 5'-noncoding region for BiP and PDGF. Recently,

10   vascular endothelial growth factor (VEGF) was also found to have IRES element (Stein et al. (1998) *Mol Cell Biol* 18:3112-3119; Huez et al. (1998) *Mol Cell Biol* 18:6178-6190).

Apart from the oligopyrimidine tract, nucleotide sequence *per se* does not appear to be important for IRES function. Without wishing to be bound by theory, a possible explanation for the function of an IRES is that it forms secondary and/or tertiary structures

15   which orient particular single-stranded regions of its sequence in a three-dimensional configuration that is conducive to interaction with a mammalian ribosome (either ribosomal protein and/or ribosomal RNA components) and/or initiation factor(s) and/or RNA binding proteins which interact with ribosomes and/or initiation factors. It is also possible that the three-dimensional structure of the IRES is determined or stabilized by one or more RNA-

20   binding proteins. Thus it is possible to devise synthetic IRES sequences having similar single-stranded regions in a similar three-dimensional configuration.

In certain cases, one or more *trans*-acting cellular proteins may be required for IRES function. For example, the HAV and entero/rhinovirus IRESes function inefficiently *in vitro* in reticulocyte lysates. Supplementation of a reticulocyte lysate with a cytoplasmic

25   extract from HeLa, Krebs II ascites, or L-cells restores activity of entero/rhinovirus IRESes. *See*, for example, Brown *et al.* (1979) *Virology* 97:396-405; and Dorner *et al.* (1984) *J. Virol.* 50:507-514. Activity of the HAV IRES *in vitro* is stimulated by liver cytoplasmic extracts. Glass *et al.* (1993) *Virology* 193:1047-1050. These observations indicate that cell-specific translational regulation can be achieved through the use of a cell-

30   specific IRES. Furthermore, coordinated cell-specific transcriptional and translational regulatory elements can be included in a vector to further increase cell specificity of viral replication. For example, the combination of an AFP-TRE and a HAV-IRES can be used to direct preferential replication of a vector in hepatic cells. Thus, in one illustrative

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embodiment, a vector comprises an AFP-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES. In another illustrative embodiment, the vector comprises a probasin-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES.

5   In yet another illustrative embodiment, a vector comprises a CMV-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES.

Examples of IRES which can be used in the present invention include those provided in Table 1 and Table 2. An IRES sequence which may be used in the present

10   invention may be tested as follows. A test vector is produced having a reporter gene, such as luciferase, for example, placed under translational control of an IRES to be tested. A desired cell type is transfected with the vector containing the desired IRES-reporter gene and an assay is performed to detect the presence of the reporter gene. In one illustrative example, the test vector comprises a co-transcribed chloramphenicol transferase (CAT) and

15   luciferase encoding gene transcriptionally driven by a CMV promoter wherein the luciferase encoding gene is translationally driven by an IRES to be tested. Host cells are transiently transfected with the test vector by means known to those of skill in the art and assayed for the presence of luciferase.

IRES may be prepared using standard recombinant and synthetic methods known in

20   the art, and as described in the Examples. For cloning convenience, restriction sites may be engineered into the ends of the IRES fragments to be used.

Transcriptional response elements (TREs)

The adenovirus vectors of the invention comprise target cell specific TREs which direct preferential expression of an operatively linked gene (or genes) in a particular target

25   cell. A TRE can be tissue-specific, tumor-specific, developmental stage-specific, cell status specific, *etc.*, depending on the type of cell present in the tissue or tumor.

Cell- and tissue-specific transcriptional regulatory elements, as well as methods for their identification, isolation, characterization, genetic manipulation and use for regulation of operatively linked coding sequences, are well known in the art. A TRE can be derived

30   from the transcriptional regulatory sequences of a single gene, or sequences from different genes can be combined to produce a functional TRE. A cell-specific TRE is preferentially functional in a limited population (or type) of cells, *e.g.*, prostate cells or liver cells. Accordingly, in some embodiments, the TRE used is preferentially functional in any of the

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following cell types: prostate; liver; breast; urothelial cells (bladder); colon; lung; ovarian; pancreas; stomach; and uterine. In other embodiments, in accordance with cell status, the TRE is functional in or during: low oxygen conditions (hypoxia); certain stages of cell cycle, such as S phase; elevated temperature; ionizing radiation.

5   As is known in the art, activity of TREs can be inducible. Inducible TREs generally exhibit low activity in the absence of inducer, and are up-regulated in the presence of inducer. Inducers include, for example, nucleic acids, polypeptides, small molecules, organic compounds and/or environmental conditions such as temperature, pressure or hypoxia. Inducible TREs may be preferred when expression is desired only at certain times

10   or at certain locations, or when it is desirable to titrate the level of expression using an inducing agent. For example, transcriptional activity from the *PSE*-TRE, *PB*-TRE and *hKLK2*-TRE is inducible by androgen, as described herein and in PCT/US98/04080. Accordingly, in one embodiment of the present invention, an adenovirus vector comprises an inducible heterologous TRE.

15   TRE multimers are also useful in the disclosed vectors. For example, a TRE can comprise a tandem series of at least two, at least three, at least four, or at least five promoter fragments. Alternatively, a TRE can comprise one or more promoter regions along with one or more enhancer regions. TRE multimers can also comprise promoter and/or enhancer sequences from different genes. The promoter and enhancer components

20   of a TRE can be in any orientation with respect to each other and can be in any orientation and/or any distance from the coding sequence of interest, as long as the desired cell-specific transcriptional activity is obtained.

        The disclosed vectors are designed such that replication is preferentially enhanced in target cells in which the TRE(s) is (are) functional. More than one TRE can be present

25   in a vector, as long as the TREs are functional in the same target cell. However, it is important to note that a given TRE can be functional in more than one type of target cell. For example, the CEA-TRE functions in, among other cell types, gastric cancer cells, colorectal cancer cells, pancreatic cancer cells and lung cancer cells.

        A TRE for use in the present vectors may or may not comprise a silencer. The

30   presence of a silencer (*i.e.*, a negative regulatory element known in the art) can assist in shutting off transcription (and thus replication) in non-target cells. Thus, presence of a silencer can confer enhanced cell-specific vector replication by more effectively preventing

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replication in non-target cells. Alternatively, lack of a silencer may stimulate replication in target cells, thus conferring enhanced target cell-specificity.

As is readily appreciated by one skilled in the art, a TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. 5 Methods of nucleotide substitution, addition, and deletion are known in the art, and readily-available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite cell-specific transcription regulatory function. Hence, functionally preserved variants of TREs, comprising nucleic acid substitutions, additions, and/or deletions, can be used in the vectors 10 disclosed herein. Accordingly, variant TREs retain function in the target cell but need not exhibit maximal function. In fact, maximal transcriptional activation activity of a TRE may not always be necessary to achieve a desired result, and the level of induction afforded by a fragment of a TRE may be sufficient for certain applications. For example, if used for treatment or palliation of a disease state, less-than-maximal responsiveness may be 15 sufficient if, for example, the target cells are not especially virulent and/or the extent of disease is relatively confined.

Certain base modifications may result in enhanced expression levels and/or cell-specificity. For example, nucleic acid sequence deletions or additions within a TRE can move transcription regulatory protein binding sites closer or farther away from each other 20 than they exist in their normal configuration, or rotate them so they are on opposite sides of the DNA helix, thereby altering spatial relationship among TRE-bound transcription factors, resulting in a decrease or increase in transcription, as is known in the art. Thus, while not wishing to be bound by theory, the present disclosure contemplates the possibility that certain modifications of a TRE will result in modulated expression levels as directed 25 by the TRE, including enhanced cell-specificity. Achievement of enhanced expression levels may be especially desirable in the case of more aggressive forms of neoplastic growth, and/or when a more rapid and/or aggressive pattern of cell killing is warranted (for example, in an immunocompromised individual).

Transcriptional activity directed by a TRE (including both inhibition and 30 enhancement) can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA and/or of a protein product encoded by the sequence under control of (*i.e.*, operably linked to) a TRE.

As discussed herein, a TRE can be of varying lengths, and of varying sequence composition. The size of a heterologous TRE will be determined in part by the capacity of the viral vector, which in turn depends upon the contemplated form of the vector (see *infra*). Generally minimal sizes are preferred for TREs, as this provides potential room for
5   insertion of other sequences which may be desirable, such as transgenes (discussed *infra*) and/or additional regulatory sequences. In a preferred embodiment, such an additional regulatory sequence is an IRES. However, if no additional sequences are contemplated, or if, for example, an adenoviral vector will be maintained and delivered free of any viral packaging constraints, larger TRE sequences can be used as long as the resultant adenoviral
10   vector remains replication-competent.

An adenoviral vector can be packaged with extra sequences totaling up to about 5% of the genome size, or approximately 1.8 kb, without requiring deletion of viral sequences. If non-essential sequences are removed from the adenovirus genome, an additional 4.6 kb of insert can be tolerated (*i.e.*, for a total insertion capacity of about 6.4 kb). Examples of
15   non-essential adenoviral sequences that can be deleted are E3, and E4 sequences other than those which encode E4 ORF6.

To minimize non-specific replication, endogenous (*e. g.*, adenovirus) TREs are preferably removed from the vector. Besides facilitating target cell-specific replication, removal of endogenous TREs also provides greater insert capacity in a vector, which may
20   be of special concern if an adenoviral vector is to be packaged within a virus particle. Even more importantly, deletion of endogenous TREs prevents the possibility of a recombination event whereby a heterologous TRE is deleted and the endogenous TRE assumes transcriptional control of its respective adenovirus coding sequences (thus allowing non-specific replication). In one embodiment, an adenoviral vector is constructed such that the
25   endogenous transcription control sequences of adenoviral genes are deleted and replaced by one or more heterologous TREs. However, endogenous TREs can be maintained in the adenovirus vector(s), provided that sufficient cell-specific replication preference is preserved. These embodiments are constructed by inserting heterologous TREs between an endogenous TRE and a replication gene coding segment. Requisite cell-specific replication
30   preference is determined by conducting assays that compare replication of the adenovirus vector in a cell which allows function of the heterologous TREs with replication in a cell which does not.

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Generally, a TRE will increase replication of a vector in a target cell by at least
about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold more
preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at
least about 100-fold, more preferably at least about 200-fold, even more preferably at least

5   about 400- to about 500- fold, even more preferably at least about 1000-fold, compared to
basal levels of replication in the absence of a TRE. The acceptable differential can be
determined empirically (by measurement of mRNA levels using, for example, RNA blot
assays, RNase protection assays or other assays known in the art) and will depend upon the
anticipated use of the vector and/or the desired result.

10   Replication-competent adenovirus vectors directed at specific target cells can be
generated using TREs that are preferentially functional in a target cell. In one embodiment
of the present invention, the target cell is a tumor cell. Non-limiting examples of tumor
cell-specific heterologous TREs, and their respective target cells, include: probasin (PB),
target cell, prostate cancer (PCT/US98/04132); α-fetoprotein (*AFP*), target cell liver cancer

15   (PCT/US98/04084); mucin-like glycoprotein DF3 (*MUC1*), target cell, breast carcinoma
(PCT/US98/04080); carcinoembryonic antigen (*CEA*), target cells, colorectal, gastric,
pancreatic, breast, and lung cancers (PCT/US98/04133); plasminogen activator urokinase
(*uPA*) and its receptor gene, target cells, breast, colon, and liver cancers
(PCT/US98/04080); *E2F1* (cell cycle S-phase specific promoter); target cell, tumors with

20   disrupted retinoblastoma gene function, and *HER-2/neu* (*c-erbB2/neu*), target cell, breast,
ovarian, stomach, and lung cancers (PCT/US98/04080); tyrosinase, target cell, melanoma
cells as described herein and uroplakins, target cell, bladder cells as described herein.
Methods for identification, isolation, characterization and utilization of additional target
cell-specific TREs are readily available to those of skill in the art.

25   In addition, tumor-specific TREs can be used in conjunction with tissue-specific
TREs from the following exemplary genes (tissue in which the TREs are specifically
functional are in parentheses): hypoxia responsive element, vascular endothelial growth
factor receptor (endothelium), albumin (liver), factor VII (liver), fatty acid synthase (liver),
Von Willebrand factor (brain endothelium), alpha-actin and myosin heavy chain (both in

30   smooth muscle), synthetase I (small intestine) Na^+-K^+-Cl^- transporter (kidney). Additional
tissue-specific TREs are known in the art.

In one embodiment of the present invention, a target cell-specific, heterologous
TRE is tumor cell-specific. A vector can comprise a single tumor cell-specific TRE or

multiple heterologous TREs which are tumor cell-specific and functional in the same cell. In another embodiment, a vector comprises one or more heterologous TREs which are tumor cell-specific and additionally comprises one or more heterologous TREs which are tissue specific, whereby all TREs are functional in the same cell.

5   Prostate-specific TREs

In one embodiment, adenovirus vectors comprise heterologous TREs that are prostate cell specific. For example, TREs that function preferentially in prostate cells and can be used to target adenovirus replication to prostate neoplasia, include, but are not limited to, TREs derived from the prostate-specific antigen gene (*PSA*-TRE) (Henderson

10   U.S. Patent No. 5,698,443); the glandular kallikrein-1 gene (from the human gene, *hKLK2*-TRE) (PCT US98/16312), and the probasin gene (*PB*-TRE) (PCT/US98/04132). All three of these genes are preferentially expressed in prostate cells and their expression is androgen-inducible. Generally, expression of genes responsive to androgen induction is mediated by an androgen receptor (AR).

15   *Prostate-specific Antigen (PSA)*

PSA is synthesized exclusively in prostatic epithelial cells and is synthesized in these cells whether they are normal, hyperplastic, or malignant. This tissue-specific expression of PSA has made it an excellent biomarker for benign prostatic hyperplasia (BPH) and prostatic carcinoma (CaP). Normal serum levels of PSA are typically below 5

20   ng/ml, with elevated levels indicative of BPH or CaP. Lundwall *et al.* (1987) *FEBS Lett.* **214**:317; Lundwall (1989) *Biochem. Biophys. Res. Comm.* **161**:1151; and Riegmann *et al.* (1991) *Molec. Endocrin.* **5**:1921.

The region of the *PSA* gene that provides androgen-dependent cell specificity, particularly in prostate cells, involves approximately 6.0 kilobases (kb). Schuur *et al.*

25   (1996) *J. Biol. Chem.* **271**:7043-7051. An enhancer region of approximately 1.5 kb in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the *PSA* gene. Within these enhancer sequences is an androgen response element (ARE) a sequence which binds androgen receptor. The sequence coordinates of the *PSA* promoter are from about nt -540 to nt +8 relative to the transcription start site. Juxtapositioning of

30   the enhancer and promoter yields a fully functional, minimal prostate-specific TRE (*PSA*-TRE). Other portions of this approximately 6.0 kb region of the *PSA* gene can be used in the vectors described herein, as long as requisite functionality is maintained.

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Human glandular Kallikrein (hKLK2)

Human glandular kallikrein (*hKLK2*, encoding the hK2 protein) is expressed exclusively in the prostate and its expression is up-regulated by androgens, primarily through transcriptional activation. Wolf *et al.* (1992) *Molec. Endocrinol.* 6:753-762;

5   Morris (1989) *Clin. Exp. Pharm. Physiol.* 16:345-351; Qui *et al.* (1990) *J. Urol.* 144:1550-1556; and Young *et al.* (1992) *Biochem.* 31:818-824. The levels of hK2 found in various tumors and in the serum of patients with prostate cancer indicate that hK2 antigen may be a significant marker for prostate cancer. Charlesworth *et al.* (1997) *Urology* 49:487-493. Expression of hK2 has been detected in each of 257 radical prostatectomy specimens

10   analyzed. Darson *et al.* (1997) *Urology* 49:857-862. The intensity and extent of hK2 expression, detected using specific antibodies, was observed to increase from benign epithelium to high-grade prostatic intraepithelial neoplasia (PIN) and adenocarcinoma.

The activity of the *hKLK2* promoter has been described and a region up to nt -2256 relative to the transcription start site was previously disclosed. Schedlich *et al.* (1987) *DNA*

15   6:429-437. The *hKLK2* promoter is androgen responsive and, in plasmid constructs wherein the promoter alone controls the expression of a reporter gene, expression of the reporter gene is increased approximately 10-fold in the presence of androgen. Murtha *et al.* (1993) *Biochem.* 32:6459-6464. *hKLK2* enhancer activity is found within a polynucleotide sequence approximately nt -12,014 to nt -2257 relative to the start of transcription and,

20   when this sequence is operably linked to an *hKLK2* promoter and a reporter gene, transcription of operably-linked sequences in prostate cells increases in the presence of androgen to levels approximately 30-fold to approximately 100-fold greater than the level of transcription in the absence of androgen. This induction is generally independent of the orientation and position of the enhancer sequences. Enhancer activity has also been

25   demonstrated in the following regions (all relative to the transcription start site): about nt -3993 to about nt -3643, about nt -4814 to about nt -3643, about nt -5155 to about nt -3387, about nt -6038 to about nt -2394.

Thus, a *hKLK2* enhancer can be operably linked to an *hKLK2* promoter or a heterologous promoter to form a *hKLK2* transcriptional regulatory element (*hKLK2*-TRE).

30   A *hKLK2*-TRE can then be operably linked to a heterologous polynucleotide to confer *hKLK2*-TRE-specific transcriptional regulation on the linked gene, thus increasing its expression.

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Probasin

The rat probasin (*PB*) gene encodes an androgen and zinc-regulated protein first characterized in the dorsolateral prostate of the rat. Dodd *et al.* (1983) *J. Biol. Chem.* 258:10731–10737; Matusik *et al.* (1986) *Biochem. Cell. Biol.* 64:601-607; and Sweetland

5   *et al.* (1988) *Mol. Cell. Biochem.* 84:3-15. The dorsolateral lobes of the murine prostate are considered the most homologous to the peripheral zone of the human prostate, where approximately 68% of human prostate cancers are thought to originate.

A *PB*-TRE has been shown to exist in an approximately 0.5 kb fragment of sequence upstream of the probasin coding sequence, from about nt -426 to about nt +28

10   relative to the transcription start site. This minimal promoter sequence from the PB gene appears to provide sufficient information to direct prostate-specific developmental- and hormone -regulated expression of an operably linked heterologous gene in transgenic mice. Greenberg *et al.* (1994) *Mol. Endocrinol.* 8:230-239.

Alpha-fetoprotein

15   α-fetoprotein (AFP) is an oncofetal protein, the expression of which is primarily restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut), although the level of its expression varies greatly depending on the tissue and the developmental stage. AFP is of clinical interest because the serum concentration of AFP is elevated in a majority of hepatoma patients, with high levels of AFP found in patients with

20   advanced disease. High serum AFP levels in patients appear to be due to AFP expression in hepatocellular carcinoma (HCC), but not in surrounding normal liver. Thus, expression of the AFP gene appears to be characteristic of hepatoma cells. An AFP-TRE is described in for example PCT/US98/04084.

According to published reports, the AFP-TRE is responsive to cellular proteins

25   (transcription factors and/or co-factor(s)) associated with AFP-producing cells, such as AFP-binding protein (see, for example, U.S. Pat. No. 5,302,698) and comprises at least a portion of an AFP promoter and/or an AFP enhancer. Cell-specific TREs from the *AFP* gene have been identified. For example, the cloning and characterization of human AFP-specific enhancer activity is described in Watanabe *et al.* (1987) *J. Biol. Chem.* 262:4812-

30   4818. A 5' *AFP* regulatory region (containing the promoter, putative silencer, and enhancer) is contained within approximately 5 kb upstream from the transcription start site.

Within the AFP regulatory region, a human *AFP* enhancer region is located between about nt -3954 and about nt -3335, relative to the transcription start site of the *AFP*

gene. The human *AFP* promoter encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two genetic elements, yields a fully functional *AFP*-TRE. Ido *et al.* (1995) *Cancer Res.* **55**:3105-3109 describe a 259 bp promoter fragment (nt -230 to nt +29) that is specific for expression in HCC cells. The *AFP* enhancer, located between

5   nt -3954 and nt -3335 relative to the transcription start site, contains two regions, denoted A and B. The promoter region contains typical TATA and CAAT boxes. Preferably, the *AFP*-TRE contains at least one enhancer region. More preferably, the *AFP*-TRE contains both enhancer regions.

Suitable target cells for vectors containing *AFP*-TREs are any cell type that allow

10   an *AFP*-TRE to function. Preferred are cells that express or produce AFP, including, but not limited to, tumor cells expressing AFP. Examples of such cells are hepatocellular carcinoma (HCC) cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto *et al.* (1992) *Hepatogastroenterology* **39**:282–286), primary gall bladder tumor

15   (Katsuragi *et al.* (1989) *Rinsko Hoshasen* **34**:371–374), uterine endometrial adenocarcinoma cells (Koyama *et al.* (1996) *Jpn. J. Cancer Res.* **87**:612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce AFP. Especially preferred as target cells for an AFP-TRE are

20   hepatocellular carcinoma cells and any of their metastases.

AFP production can be measured (and hence AFP-producing cells can be identified) using immunoassays standard in the art, such as RIA, ELISA or protein immunoblotting (Western blots) to determine levels of AFP protein production; and/or RNA blotting (Northern blots) to determine AFP mRNA levels. Alternatively, such cells can be

25   identified and/or characterized by their ability to activate transcriptionally an *AFP*-TRE (*i.e.*, allow an *AFP*-TRE to function).

See also co-owned PCT WO98/39465 regarding AFP-TREs. As described in more detail therein, an AFP-TRE can comprise any number of configurations, including, but not limited to, an AFP promoter; an AFP enhancer; an AFP promoter and an AFP enhancer; an

30   AFP promoter and a heterologous enhancer; a heterologous promoter and an AFP enhancer; and multimers of the foregoing. The promoter and enhancer components of an AFP-TRE can be in any orientation and/or distance from the coding sequence of interest, as long as the desired AFP cell-specific transcriptional activity is obtained. An adenovirus

vector of the present invention can comprise an AFP-TRE endogenous silencer element or the AFP-TRE endogenous silencer element can be deleted.

Urokinase Plasminogen Activator

The protein urokinase plasminogen activator (uPA) and its cell surface receptor, urokinase plasminogen activator receptor (uPAR), are expressed in many of the most frequently-occurring neoplasms and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast, colon, prostate, liver, renal, lung and ovarian cancer. Sequence elements that regulate uPA and uPAR transcription have been extensively studied. Riccio *et al.* (1985) *Nucleic Acids Res.* **13**:2759-2771; Cannio *et al.* (1991) *Nucleic Acids Res.* **19**:2303–2308.

Carcinoembryonic antigen (CEA)

CEA is a 180,000 Dalton, tumor-associated, glycoprotein antigen present on endodermally-derived neoplasms of the gastrointestinal tract, such as colorectal, gastric (stomach) and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because circulating CEA can be detected in the great majority of patients with CEA-positive tumors. In lung cancer, about 50% of total cases have circulating CEA, with high concentrations of CEA (greater than 20 ng/ml) often detected in adenocarcinomas. Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

The 5'-flanking sequence of the *CEA* gene has been shown to confer cell-specific activity. The *CEA* promoter region, approximately the first 424 nucleotides upstream of the transcriptional start site in the 5' flanking region of the gene, was shown to confer cell-specific activity by virtue of providing higher promoter activity in CEA-producing cells than in non-producing HeLa cells. Schrewe *et al.* (1990) *Mol. Cell. Biol.* **10**:2738-2748. In addition, cell-specific enhancer regions have been found. *See* PCT/GB/02546 The *CEA* promoter, putative silencer, and enhancer elements appears to be contained within a region that extends approximately 14.5 kb upstream from the transcription start site. Richards et al. (1995); PCT/GB/02546. Further characterization of the 5'-flanking region of the *CEA* gene by Richards *et al.* (1995) *supra* indicated that two upstream regions (one between about -13.6 and about -10.7 kb, and the other between about -6.1 and about-4.0 kb), when linked to the multimerized promoter, resulted in high-level and selective expression of a reporter construct in CEA-producing LoVo and SW1463 cells. Richards et al. (1995) *supra* also localized the promoter region between about nt -90 and

about nt +69 relative to the transcriptional start site, with the region between about nt -41
and about nt -18 being essential for expression. PCT/GB/02546 describes a series of
5'-flanking *CEA* fragments which confer cell-specific activity, including fragments
comprising the following sequences: about nt -299 to about nt +69; about nt -90 to about

5   nt +69; nt -14,500 to nt -10,600; nt -13,600 to nt -10,600; and nt -6100 to nt -3800, with all
coordinates being relative to the transcriptional start point. In addition, cell-specific
transcription activity is conferred on an operably linked gene by the *CEA* fragment from
nt -402 to nt +69.

       CEA-TREs for use in the vectors disclosed herein are derived from mammalian

10   cells, including, but not limited to, human cells. Thus, any of the *CEA*-TREs can be used
as long as the requisite desired functionality is displayed by the vector.

       <u>Mucin</u>

       The protein product of the *MUC1* gene (known as mucin, MUC1 protein; episialin;
polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3

15   antigen) is normally expressed mainly at the apical surface of epithelial cells lining the
glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands,
and mammary glands. Zotter *et al.* (1988) *Cancer Rev.* **11–12**:55–101; and Girling *et al.*
(1989) *Int. J. Cancer* **43**:1072–1076. However, mucin is overexpressed in 75–90% of
human breast carcinomas. Kufe *et al.* (1984) *Hybridoma* **3**:223–232. For reviews, see

20   Hilkens (1988) *Cancer Rev.* **11–12**:25–54; and Taylor-Papadimitriou, *et al.* (1990) *J. Nucl.*
Med. Allied Sci. **34**:144–150. Mucin protein expression correlates with the degree of breast
tumor differentiation. Lundy *et al.* (1985) *Breast Cancer Res. Treat.* **5**:269–276.

       Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and
ZR-75-1 appears to occur at the transcriptional level. Kufe *et al.* (1984) *supra*; Kovarik

25   (1993) *J. Biol. Chem.* **268**:9917–9926; and Abe *et al.* (1990) *J. Cell. Physiol.* **143**:226–
231. The regulatory sequences of the *MUC1* gene have been cloned, including the
approximately 0.9 kb upstream of the transcription start site which contains a TRE that
appears to be involved in cell-specific transcription. Abe *et al.* (1993) *Proc. Natl. Acad.*
Sci. USA **90**:282-286; Kovarik et al. (1993) *supra*; and Kovarik *et al.* (1996) *J. Biol.*

30   *Chem.* **271**:18140–18147.

       MUC1-TREs are derived from mammalian cells, including but not limited to,
human cells. Preferably, the *MUC1*-TRE is human. In one embodiment, the *MUC1*-TRE
contains the entire 0.9 kb 5' flanking sequence of the *MUC1* gene. In other embodiments,

MUC1-TREs comprise the following sequences (relative to the transcription start site of the *MUC1* gene) operably-linked to a promoter: about nt -725 to about nt +31, about nt -743 to about nt +33, about nt -750 to about nt +33, and about nt -598 to about nt +485.

c-erbB2/*HER-2*/neu

5   The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a transforming gene that encodes a 185 kD epidermal growth factor receptor-related transmembrane glycoprotein. In humans, the c-erbB2/neu protein is expressed during fetal development and, in adults, the protein is weakly detectable (by immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or over-expression of the *c-erbB2/neu* gene has been associated with

10   many human cancers, including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical consequences of overexpression of the c-erbB2/neu protein have been best studied in breast and ovarian cancer. c-erbB2/neu protein over-expression occurs in 20 to 40% of intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a poor prognosis in subcategories of both diseases.

15   Human, rat and mouse *c-erbB2/neu* TREs have been identified and shown to confer transcriptional activity specific to *c-erbB2/neu*-expressing cells. Tal *et al.* (1987) *Mol. Cell. Biol.* 7:2597–2601; Hudson *et al.* (1990) *J. Biol. Chem.* 265:4389–4393; Grooteclaes *et al.* (1994) *Cancer Res.* 54:4193–4199; Ishii *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4374–4378; and Scott *et al.* (1994) *J. Biol. Chem.* 269:19848–19858.

20   Melanocyte-specific TRE

It has been shown that some genes which encode melanoma proteins are frequently expressed in melanoma/melanocytes, but silent in the majority of normal tissues. A variety of melanocyte-specific TRE are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with melanocytes, and comprise at least a portion of

25   a melanocyte-specific promoter and/or a melanocyte-specific enhancer. Known transcription factors that control expression of one or more melanocyte-specific genes include the microphthalmia associated transcription factor MITF. Yasumoto et al. (1997) *J. Biol. Chem.* 272:503-509. Other transcription factors that control expression of one or more melanocyte specific genes include MART-1/Melan-A, gp100, TRP-1 and TRP-2

30   Methods are described herein for measuring the activity of a melanocyte-specific TRE and thus for determining whether a given cell allows a melanocyte-specific TRE to function.

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In some embodiments, the melanocyte-specific TREs used in this invention are derived from mammalian cells, including but not limited to, human, rat, and mouse. Any melanocyte-specific TREs may be used in the adenoviral vectors of the invention. Rodent and human 5' flanking sequences from genes expressed specifically or preferentially in

5   melanoma cells have been described in the literature and are thus made available for practice of this invention and need not be described in detail herein. The following are some examples of melanocyte-specific TREs which can be used. A promoter and other control elements in the human tyrosinase gene 5' flanking region have been described and sequences have been deposited as GenBank Accession Nos. X16073 and D10751. Kikuchi

10   et al. (1989) *Biochim. Biophys. Acta* 1009:283-286; and Shibata et al. (1992) *J. Biol. Chem.* 267:20584-20588. A cis-acting element has been defined that enhances melanocyte-specific expression of human tyrosinase gene. This element comprises a 20-bp sequence known as tyrosinase distal element (TDE), contains a CATGTG motif, and lies at positions about -1874 to about -1835 relative to the human tyrosinase gene transcription start site.

15   Yasumoto et al. (1994) *Mol. Cell. Biol.* 14:8058-8070. A promoter region comprising sequences from about -209 to +61 of the human tyrosinase gene was found to direct melanocyte-specific expression. Shibata (1992). Similarly, the mouse tyrosinase 5' flanking region has been analyzed and a sequence deposited as GenBank Accession Nos. D00439 and X51743. Klüppel et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:3777-3788. A

20   minimal promoter has been identified for the mouse TRP-1 gene, and was reported to encompass nucleotides -44 to +107 relative to the transcription start site. Lowings et al. (1992) *Mol. Cell. Biol.* 12:3653-3662. Two regulatory regions required for melanocyte-specific expression of the human TRP-2 gene have been identified. Yokoyama et al. (1994) *J. Biol. Chem.* 269:27080-27087. A human MART-1 promoter region has been

25   described and deposited as GenBank Accession No. U55231. Melanocyte-specific promoter activity was found in a 233-bp fragment of the human MART-1 gene 5' flanking region. Butterfield et al. (1997) *Gene* 191:129-134. A basic-helix-loop-helix/leucine zipper-containing transcription factor, MITF (microphthalmia associated transcription factor) was reported to be involved in transcriptional activation of tyrosinase and TRP-1

30   genes. Yasumoto et al. (1997) *J. Biol. Chem.* 272:503-509.

In some embodiments, a melanocyte-specific TRE comprises sequences derived from the 5' flanking region of a human tyrosinase gene depicted in Table 3. In some of these embodiments, the melanocyte-specific TRE comprises tyrosinase nucleotides from

about -231 to about +65 relative to the transcription start site (from about nucleotide 244 to about nucleotide 546 of SEQ ID NO:10) and may further comprise nucleotides from about -1956 to about -1716 relative to the human tyrosinase transcription start site (from about nucleotide 6 to about nucleotide 243 of SEQ ID NO:10). A melanocyte-specific TRE can

5 comprise nucleotides from about ---231 to about + 65 juxtaposed to nucleotides from about -1956 to about -1716. It has been reported that nucleotides from about -1956 to about -1716 relative to the human tyrosinase transcription start site can confer melanocyte-specific expression of an operably linked reporter gene with either a homologous or a heterologous promoter. Accordingly, in some embodiments, a melanocyte-specific TRE comprises

10 nucleotides from about -1956 to about -1716 operably linked to a heterologous promoter.

A melanocyte-specific TRE can also comprise multimers. For example, a melanocyte-specific TRE can comprise a tandem series of at least two, at least three, at least four, or at least five tyrosinase promoter fragments. Alternatively, a melanocyte-specific TRE could have one or more tyrosinase promoter regions along with one or more

15 tyrosinase enhancer regions. These multimers may also contain heterologous promoter and/or enhancer sequences.

Cell status-specific TREs

Cell status-specific TREs for use in the adenoviral vectors of the present invention can be derived from any species, preferably a mammal. A number of genes have been

20 described which are expressed in response to, or in association with, a cell status. Any of these cell status-associated genes may be used to generate a cell status-specific TRE.

An example of a cell status is cell cycle. An exemplary gene whose expression is associated with cell cycle is E2F-1, a ubiquitously expressed, growth-regulated gene, which exhibits peak transcriptional activity in S phase. Johnson et al. (1994) *Genes Dev.* 8:1514-

25 1525. The RB protein, as well as other members of the RB family, form specific complexes with E2F-1, thereby inhibiting its ability to activate transcription. Thus, E2F-1-responsive promoters are down-regulated by RB. Many tumor cells have disrupted RB function, which can lead to de-repression of E2F-1-responsive promoters, and, in turn, de-regulated cell division.

30 Accordingly, in one embodiment, the invention provides an E3-containing adenoviral vector in which an adenoviral gene (preferably a gene necessary for replication) is under transcriptional control of a cell status-specific TRE, wherein the cell status-specific

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TRE comprises a cell cycle-activated TRE. In one embodiment, the cell cycle-activated
TRE is an E2F1 TRE.

Another group of genes that are regulated by cell status are those whose expression
is increased in response to hypoxic conditions. Bunn and Poyton (1996) *Physiol. Rev.*
5   76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemin and
Krasnow (1997) *Cell* 89:9-12. Many tumors have insufficient blood supply, due in part to
the fact that tumor cells typically grow faster than the endothelial cells that make up the
blood vessels, resulting in areas of hypoxia in the tumor. Folkman (1989) *J. Natl. Cancer
Inst.* 82:4-6; and Kallinowski (1996) *The Cancer J.* 9:37-40. An important mediator of
10   hypoxic responses is the transcriptional complex HIF-1, or hypoxia inducible factor-1,
which interacts with a hypoxia-responsive element (HRE) in the regulatory regions of
several genes, including vascular endothelial growth factor, and several genes encoding
glycolytic enzymes, including enolase-1. Murine HRE sequences have been identified and
characterized. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500. An HRE from
15   a rat enolase-1 promoter is described in Jiang et al. (1997) *Cancer Res.* 57:5328-5335. An
HRE from a rat enolase-1 promoter is depicted in Table 3.

Accordingly, in one embodiment, an adenovirus vector comprises an adenovirus
gene, preferably an adenoviral gene essential for replication, under transcriptional control
of a cell status-specific TRE comprising an HRE. In one embodiment, the cell status-
20   specific TRE comprises the HRE depicted in Table 3.

Other cell status-specific TREs include heat-inducible (i.e., heat shock) promoters,
and promoters responsive to radiation exposure, including ionizing radiation and UV
radiation. For example, the promoter region of the early growth response-1 (Egr-1) gene
contains an element(s) inducible by ionizing radiation. Hallahan et al. (1995) *Nat. Med.*
25   1:786-791; and Tsai-Morris et al. (1988) *Nucl. Acids. Res.* 16:8835-8846. Heat-inducible
promoters, including heat-inducible elements, have been described. See, for example
Welsh (1990) in "Stress Proteins in Biology and Medicine", Morimoto, Tisseres, and
Georgopoulos, eds. Cold Spring Harbor Laboratory Press; and Perisic et al. (1989) *Cell*
59:797-806. Accordingly, in some embodiments, the cell status-specific TRE comprises an
30   element(s) responsive to ionizing radiation. In one embodiment, this TRE comprises a 5'
flanking sequence of an Egr-1 gene. In other embodiments, the cell status-specific TRE
comprises a heat shock responsive element.

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The cell status-specific TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell status-specific TREs are known in the art, as are methods to identify and test cell status specificity of suspected cell status-specific TREs.

5   *Urothelial cell-specific TREs*

Any urothelial cell-specific TRE may be used in the adenoviral vectors of the invention. A number of urothelial cell-specific proteins have been described, among which are the uroplakins. Uroplakins (UP), including UPIa and UPIb (27 and 28 kDa, respectively), UPII (15 kDa), and UPIII (47 kDa), are members of a group of integral

10   membrane proteins that are major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian urothelium and may play a role as a permeability barrier and/or as a physical stabilizer of the urothelial apical surface. Wu et al. (1994) *J. Biol. Chem.* 269:13716-13724. UPs are bladder-specific proteins, and are expressed on a significant proportion of urothelial-derived tumors, including about 88% of

15   transitional cell carcinomas. Moll et al. (1995) *Am. J. Pathol.* 147:1383-1397; and Wu et al. (1998) *Cancer Res.* 58:1291-1297. The control of the expression of the human UPII has been studied, and a 3.6-kb region upstream of the mouse UPII gene has been identified which can confer urothelial-specific transcription on heterologous genes (Lin et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:679-683).

20   Preferred urothelial cell-specific TREs include TREs derived from the uroplakins UPIa, UPIb, UPII, and UPIII, as well as urohingin. A uroplakin TRE may be from any species, depending on the intended use of the adenovirus, as well as the requisite functionality is exhibited in the target or host cell. Significantly, adenovirus constructs comprising a urothelial cell-specific TREs have observed that such constructs are capable

25   of selectively replicating in urothelial cells as opposed to smooth muscle cells, which adjoin urothelial cells in the bladder.

          Uroplakin

Urothelial-specific TREs derived from the *hUPII* gene are described herein. Accordingly, in some embodiments, an adenovirus vector of the invention comprises an

30   adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises the 2.2 kb sequence from the 5' flanking region of hUPII gene, as shown in Table 3. In other embodiments, an adenovirus vector of the invention comprises an adenovirus gene,

preferably an adenoviral gene essential for replication, under transcriptional control of a
urothelial cell-specific TRE which comprises a 1.8 kb sequence from the 5' flanking region
of hUPII gene, from nucleotides 430 to 2239 as shown in Table 3. In other embodiments,
the urothelial cell-specific TRE comprises a functional portion of the 2.2 kb sequence

5   depicted in Table 3, or a functional portion of the 1.8 kb sequence of nucleotides 430 to
2239 of the sequence depicted in Table 3, such as a fragment of 2000 bp or less, 1500 bp or
less, or 1000 bp or less, 600 bp less, or at least 200 bp which includes the 200 bp fragment
of the hUPII 5'-flanking region.

A 3.6 kb 5'-flanking sequence located from the mouse UPII (mUPII) gene which

10   confers urothelial cell-specific transcription on heterologous genes is one urothelial cell-
specific TRE useful in vectors of the instant invention (Table 3). Smaller TREs (i.e., 3500
bp or less, more preferably less than about 2000 bp, 1500 bp, or 1000 bp) are preferred.
Smaller TREs derived from the mUPII 3.6 kb fragment are one group of preferred
urothelial cell-specific TREs. In particular, Inventors have identified an approximately 600

15   bp fragment from the 5' flanking DNA of the mUPII gene, which contains 540 bp of 5'
untranslated region (UTR) of the mUPII gene, that confers urothelial cell-specific
expression on heterologous genes.

Accordingly, in some embodiments, an adenovirus vector of the invention
comprises an adenovirus gene, preferably an adenoviral gene essential for replication,

20   under transcriptional control of a urothelial cell-specific TRE which comprises the 3.6 kb
sequence from the 5' flanking region of mouse UPII gene, as shown in Table 3. In other
embodiments, the urothelial cell-specific TRE comprises a functional portion of the 3.6 kb
sequence depicted in Table 3, such as a fragment of 3500 bp or less, 2000 bp or less, 1500
bp or less, or 1000 bp or less which includes the 540 bp fragment of 5' UTR. The

25   urothelial cell-specific TRE may also be a sequence which is substantially identical to the
3.6 kb mUPII 5'-flanking region or any of the described fragments thereof.

As an example of how urothelial cell-specific TRE activity can be determined, a
polynucleotide sequence or set of such sequences can be generated using methods known
in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant

30   methods. The sequence(s) to be tested is inserted into a vector containing an appropriate
reporter gene, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-
galactosidase (encoded by the lacZ gene), luciferase (encoded by the luc gene), a green
fluorescent protein, alkaline phosphatase, and horse radish peroxidase. Such vectors and

assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative target cell-specific TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes

5　(lipofection) and DEAE dextran. Suitable host cells include any urothelial cell type, including but not limited to, KU-1, MYP3 (a non-tumorigenic rat urothelial cell line), 804G (rat bladder carcinoma cell line), cultured human urothelial cells (HUC), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. Non-urothelial cells, such as LNCaP, HBL-100, HLF, HLE, 3T3, Hep3B, HuH7, CADO-LC9, and HeLa are used as a control.

10　Results are obtained by measuring the level of expression of the reporter gene using standard assays. Comparison of expression between urothelial cells and control indicates presence or absence of transcriptional activation.

　　Comparisons between or among various urothelial cell-specific TREs can be assessed by measuring and comparing levels of expression within a single urothelial cell

15　line. It is understood that absolute transcriptional activity of a urothelial cell-specific TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of the urothelial cell-specific TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of

20　transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

25　Alternatively a putative urothelial cell-specific TRE can be assessed for its ability to confer adenoviral replication preference for cells that allow a urothelial cell-specific TRE to function. For this assay, constructs containing an adenovirus gene essential to replication operatively linked to a putative urothelial cell-specific TRE are transfected into urothelial cells. Viral replication in those cells is compared, for example, to viral

30　replication by wild type adenovirus in those cells and/or viral replication by the construct in non-urothelial cells.

TRE configurations

A TRE as used in the present invention can be present in a variety of configurations. A TRE can comprise multimers. For example, a TRE can comprise a tandem series of at least two, at least three, at least four, or at least five target cell-specific TREs. These
5   multimers may also contain heterologous promoter and/or enhancer sequences.

Optionally, a transcriptional terminator or transcriptional "silencer" can be placed upstream of the target cell-specific TRE, thus preventing unwanted read-through transcription of the coding segment under transcriptional control of the target cell-specific TRE. Also, optionally, the endogenous promoter of the coding segment to be placed under
10   transcriptional control of the target cell-specific TRE can be deleted.

A target cell-specific TRE may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) may assist in shutting off transcription (and thus replication) in non-permissive cells (i.e., a non-target cell). Thus, presence of a silencer may confer enhanced target cell-specific replication by more effectively preventing
15   adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced target cell-specific replication due to more effective replication in target cells.

It is also understood that the invention includes a target cell-specific TRE regulating the transcription of a bicistronic mRNA in which translation of the second mRNA is
20   associated by an IRES. An adenovirus vector may further include an additional heterologous TRE which may or may not be operably linked to the same gene(s) as the target cell-specific TRE. For example a TRE (such as a cell type-specific or cell status-specific TRE) may be juxtaposed to a second type of target-cell-specific TRE. "Juxtaposed" means a target cell-specific TRE and a second TRE transcriptionally control
25   the same gene. For these embodiments, the target cell-specific TRE and the second TRE may be in any of a number of configurations, including, but not limited to, (a) next to each other (i.e., abutting); (b) both 5' to the gene that is transcriptionally controlled (i.e., may have intervening sequences between them); (c) one TRE 5' and the other TRE 3' to the gene.

30   As is readily appreciated by one skilled in the art, a target cell-specific TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene

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assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite target cell-specific transcription function. Hence, the invention also includes functionally-preserved variants of the TRE nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. The variants of the

5   sequences disclosed herein may be 80%, 85%, 90%, 95%, 98%, 99% or more identical, as measured by, for example, ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using efault parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2 to any of the urothelial cell-specific TRE sequences disclosed herein. Variants of target cell-specific TRE sequences may also hybridize at high

10   stringency, that is at 68°C and 0.1X SSC, to any of the target cell-specific TRE sequences disclosed herein.

In terms of hybridization conditions, the higher the sequence identity required, the more stringent are the hybridization conditions if such sequences are determined by their ability to hybridize to a sequence of TRE disclosed herein. Accordingly, the invention also

15   includes polynucleotides that are able to hybridize to a sequence comprising at least about 15 contiguous nucleotides (or more, such as about 25, 35, 50, 75 or 100 contiguous nucleotides) of a TRE disclosed herein. The hybridization conditions would be stringent, i.e., 80°C (or higher temperature) and 6M SSC (or less concentrated SSC). Another set of stringent hybridization conditions is 68°C and 0.1 X SSC. For discussion regarding

20   hybridization reactions, see below.

Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction of widely known and published in the art. See, for example, Sambrook et al. (1989) at page 7.52. Examples of relevant conditions include (in order of increasing stringency): incubation

25   temperatures of 25°C, 37°C, 50°C and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 X SSC, 1 X SSC, 0.1 X

30   SSC, or deionized water. An exemplary set of stringent hybridization conditions is 68°C and 0.1 X SSC.

"T_m" is the temperature in degrees Celcius at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in anti-parallel direction by Watson-

Crick base pairing dissociates into single strands under conditions of the experiment. T_m may be predicted according to a standard formula, such as:

$$T_m = 81.5 + 16.6 \log[X^+] + 0.41\ (\%G/C) - 0.61\ (\%F) - 600/L$$

5

where $[X^+]$ is the cation concentration (usually sodium ion, Na^+) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

10   While not wishing to be bound by a single theory, the inventors note that it is possible that certain modifications will result in modulated resultant expression levels, including enhanced expression levels. Achievement of modulated resultant expression levels, preferably enhanced expression levels, may be especially desirable in the case of certain, more aggressive forms of cancer, or when a more rapid and/or aggressive pattern of

15   cell killing is warranted (due to an immunocompromised condition of the individual, for example).

Determination of TRE activity

Activity of a TRE can be determined, for example, as follows. A TRE polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant

20   methods. The sequence(s) to be tested can be inserted into a vector containing a promoter (if no promoter element is present in the TRE) and an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the *lacZ* gene), luciferase (encoded by the *luc* gene), alkaline

25   phosphatase (AP), green fluorescent protein (GFP), and horseradish peroxidase (HRP). Such vectors and assays are readily available, from, *inter alia*, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes, DEAE

30   dextran-mediated transfer, particle bombardment or direct injection. TRE activity is measured by detection and/or quantitation of reporter gene-derived mRNA and/or protein. Reporter protein product can be detected directly (*e.g.*, immunochemically) or through its enzymatic activity, if any, using an appropriate substrate. Generally, to determine cell

50

specific activity of a TRE, a TRE-reporter gene construct is introduced into a variety of cell types. The amount of TRE activity is determined in each cell type and compared to that of a reporter gene construct lacking the TRE. A TRE is determined to be cell-specific if it is preferentially functional in one cell type, compared to a different type of cell.

5   *Adenovirus early genes*

         The adenovirus vectors of the invention comprise two or more genes which are co-transcribed under the control of a target cell-specific TRE wherein the second gene is under translational control of an IRES. One or more of the genes can be an adenovirus gene, preferably an adenovirus gene essential for replication. Any gene that is essential for

10   adenovirus replication, such as E1A, E1B, E2, E4 or any of the late genes, is useful. The adenovirus may also comprise E3. In addition, one or more of the genes can be a transgene or heterologous gene. Any of the various adenovirus serotypes can be used, such as, for example, Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, the Ad5 serotype is exemplified herein.

15   The E1A gene is expressed immediately (between 0 and 2 hours) after viral infection, before any other viral genes. E1A protein is a *trans*-acting positive transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are also

20   expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* **651**:175–208; Flint (1986) *Advances Virus Research* **31**:169–228; and Grand (1987) *Biochem. J.* **241**:25–38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced. Nevins (1989) *Adv. Virus Res.* **31**:35–81. The transcription start site of Ad5 E1A is at

25   coordinate 498 and the ATG start site of the E1A protein is at coordinate 560 in the virus genome.

         The E1B protein is necessary in *trans* for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been

30   implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey *et al.* (1993) *Virology* **193**:631; Bailey *et al.* (1994) *Virology* **202**:695-706. The E1B promoter

of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box, and extends from Ad5 nt 1636 to 1701.

Adenovirus E1B 19-kDa (19K) protein is a potent inhibitor of apoptosis and cooperates with E1A to produce oncogenic transformation of primary cells (Rao, et al.,

5   1992, *Cell Biology*, 89:7742-7746). During productive adenovirus infection, E1A stimulates host cell DNA synthesis, thereby causing cells to aberrantly go through the cell cycle. In response to cell cycle deregulation, the host cell undergoes apoptosis. As a defense mechanism, the E1B 19-kDa protein inhibits this E1A-induced apoptosis and allows assembly of viral progeny to be completed before the cell commits suicide. E1B 19-

10   kDa conducts anti-apoptotic function by multiple mechanisms. E1B 19-kDa inhibits the apoptosis of multiple stimuli, including E1a, p53 and TNF, for example. According to wild-type Ad5 , the E1B 19-kDa region is located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao *et al., Proc. Natl. Acad. Sci. USA, 89:*7742-7746.

15   In a preferred embodiment, expression of the E1A and E1B regions of the Ad genome is facilitated in a cell-specific fashion by placing a cell-specific TRE upstream of E1A and a internal ribosome entry site between E1A and E1B.

The E2 region of adenovirus encodes proteins related to replication of the adenoviral genome, including the 72 kD DNA-binding protein, the 80 kD precursor

20   terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

25   The E2 early promoter of Ad5 is located between nucleotides 27,050 and 27,150, and consists of a major and a minor transcription initiation site (the latter accounting for about 5% of E2 transcripts), two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site. For a detailed review of E2 promoter architecture see Swaminathan *et al.* (1995) *Curr. Topics in Micro. and Imm.* **199**

30   part 3:177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps by only a few base pairs with sequences on the counterstrand

which encode a 33 kD protein. Notably, an SpeI restriction site (Ad5 position 27,082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA box from the upstream E2F and ATF binding sites. Therefore, insertion of a heterologous TRE having SpeI ends into the
5   SpeI site disrupts the endogenous E2 early promoter of Ad5 and allows TRE-regulated expression of E2 transcripts.

An E3 region refers to the region of the adenoviral genome that encodes the E3 products. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. Generally,
10   the E3 region is located between about 28583 and about 30470 of the adenoviral genome. An E3 region for use in the present invention may be from any adenovirus serotype. An E3 sequence is a polynucleotide sequence that contains a sequence from an E3 region. In some embodiments, the sequence encodes ADP. In other embodiments, the sequence encodes other than ADP and excludes a sequence encoding only ADP. As is well known in
15   the art, the ADP coding region is located in the E3 region within the adenoviral genome from about 29468 bp to about 29773 bp; including the Y leader, the location of ADP is from about 28375 bp to about 29773 bp for Ad5. Other ADP regions for other serotypes are known in the art. An E3 sequence includes, but is not limited to, deletions; insertions; fusions; and substitutions. An E3 sequence may also comprise an E3 region or a portion of
20   the E3 region. It is understood that, as an "E3 sequence" is not limited to an "E3 region", alternative references herein to an "E3 region" or "E3 sequence" do not indicate that these terms are interchangeable. Assays for determining a functional E3 sequence for purposes of this invention are described herein.

The E4 gene has a number of transcription products and encodes two polypeptides
25   (the products of open reading frames (ORFs) 3 and 6) which are responsible for stimulating the replication of viral genomic DNA and stimulating late gene expression, through interaction with heterodimers of cellular transcription factors E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55 kD protein for activity while the ORF 3 protein does not. In the absence of functional ORF 3- and ORF 6-encoded proteins, efficiency of
30   plaque formation is less than 10^{-6} that of wild type virus.

To further increase cell-specificity of replication, it is possible to take advantage of the interaction between the E4 ORF 6 gene product and the E1B 55 kD protein. For example, if E4 ORFs 1-3 are deleted, viral DNA replication and late gene synthesis

becomes dependent on E4 ORF6 protein. By generating such a deletion in a vector in which the E1B region is regulated by a cell-specific TRE, a virus is obtained in which both E1B and E4 functions are dependent on the cell-specific TRE which regulates E1B.

Late genes relevant to the disclosed vectors are L1, L2 and L3, which encode

5   proteins of the virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. All late genes are under the control of the major late promoter (MLP), which is located in Ad5 between nucleotides 5986 and 6048.

In one embodiment, an adenovirus early gene is under transcriptional control of a cell specific, heterologous TRE. In additional embodiments, the early gene is selected from

10   the group including E1A, E1B, E2, E3, E4. In another embodiment, an adenovirus late gene is under transcriptional control of a cell specific, heterologous TRE. In further embodiments, two or more early genes are under the control of heterologous TREs that function in the same target cell. The heterologous TREs can be the same or different, or one can be a variant of the other. In additional embodiments, two or more late genes are

15   under the control of heterologous TREs that function in the same target cell. The heterologous TREs can be the same or different, or one can be a variant of the other. In yet another embodiment, one or more early gene(s) and one or more late gene(s) are under transcriptional control of the same or different heterologous TREs, wherein the TREs function in the same target cell.

20   In some embodiments of the present invention, the adenovirus vector comprises the essential gene E1A and the E1A promoter is deleted. In other embodiments, the adenovirus vector comprises the essential gene E1A and the E1A enhancer I is deleted. In yet other embodiments, the E1A promoter is deleted and E1A enhancer I is deleted. In other embodiments, an internal ribosome entry site (IRES) is inserted upstream of E1B (so that

25   E1B is translationally linked), and a target cell-specific TRE is operably linked to E1A. In still other embodiments, an (IRES) is inserted upstream of E1B (so that E1B is translationally linked), and target cell-specific TRE is operably linked to E1A, which may or may not maintain the E1A promoter and/or enhancer I (i.e., the E1A promoter and/or enhancer I may be, but not necessarily be, deleted). In yet other embodiments, the 19-kDa

30   region of E1B is deleted.

For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter does not interfere with transcription of the second

gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an
initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred
that the initiation codon of the second gene is removed and that the IRES and second gene
are in frame. Alternatively, if the IRES does not contain an initiation codon or if the

5   initiation codon is removed from the IRES, the initiation codon of the second gene is used.

Adenovirus death protein (ADP) gene and gene product

        In the construction of adenovirus vectors, the E3 region is often deleted to facilitate
insertion of one or more TREs and/or transgenes. In some embodiments, however, the
adenovirus death protein (ADP), encoded within the E3 region, is retained in an adenovirus

10   vector. The ADP gene, under control of the major late promoter (MLP), appears to code
for a protein (ADP) that is important in expediting host cell lysis. Tollefson *et al.* (1992) *J.
Virol.* **66**:3633; and Tollefson *et al.* (1996) *J. Virol.* **70**:2296. Thus, inclusion of an ADP
gene in a viral vector can render the vector more potent, making possible more effective
treatment and/or a lower dosage requirement.

15   An ADP coding sequence is obtained preferably from Ad2 (since this is the strain in
which the ADP has been most fully characterized) using techniques known in the art, such
as PCR. Preferably, the Y leader (which is an important sequence for correct expression of
late genes) is also obtained and placed in operative linkage to the ADP coding sequence.
The ADP coding sequence (with or without the Y leader) is then introduced into an

20   adenoviral genome, for example, in the E3 region, where expression of the ADP coding
sequence will be driven by the MLP. The ADP coding sequence can, of course, also be
inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively,
the ADP coding sequence can be operably linked to a heterologous TRE, including, but not
limited to, another viral TRE or a target cell-specific TRE (see *infra*). In another

25   embodiment, the ADP gene is present in a viral genome such that it is transcribed as part of
a multi-cistronic mRNA in which its translation is associated with an IRES.

E3-containing target cell-specific adenoviral vectors

        In some embodiments, the adenovirus vectors contain an E3 region, or a portion of
an E3 region. Inclusion of the E3 region of adenovirus can enhance cytotoxicity of the

30   target cell-specific adenoviral vectors of the present invention. Adenoviral vectors
containing an E3 region may maintain their high level of specificity and can be (a)
significantly more cytotoxic; (b) produce higher virus yield including extracellular virus
yield; (c) form larger plaques; (d) produce rapid cell death; and (e) kill tumors more

efficiently *in vivo* than vectors lacking the E3 region. The adenoviral vectors of this invention may contain the E3 region or a portion of the E3 region. It is understood that, as inclusion of E3 confers observable and measurable functionality on the adenoviral vectors, for example, increased replication and production, functionally equivalent (in which

5   functionality is essentially maintained, preserved, or even enhanced or diminished) variants of E3 may be constructed. For example, portions of E3 may be used. A portion may be, non-inclusively, either of the following: (a) deletion, preferably at the 3' end; (b) inclusion of one or more various open reading frames of E3. Five proteins which are encoded by the Ad-E3 region have been identified and characterized: (1) a 19-kDa glycoprotein (gp19k) is

10   one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and inflammatory responses mediated by tumor necrosis factor (TNF); (3) E3 10.4k/14.5k

15   protein complex down regulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. The functions of three E3-encoded proteins -- 3.6k, 6.7k and 12.5k -- are unknown. A ninth protein having a molecular weight

20   of 7.5 kDa has been postulated to exist, but has not been detected in cells infected with wild-type adenovirus. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. The E3 region is schematically depicted in FIG. 6. These intact, portions, or variants of E3 may be readily constructed using standard knowledge and techniques in the art. Preferably, an intact E3 region is used.

25   In the adenovirus vectors of the present invention, E3 may or may not be under transcriptional control of native adenoviral transcriptional control element(s). The E3 promoter is located within the coding sequence for virion protein VIII, an essential protein which is highly conserved among adenovirus serotypes. In some embodiments, E3 is under transcriptional control of a heterologous TRE, including, but not limited to, a target cell-

30   specific TRE. Accordingly, in one embodiment, the invention provides an adenoviral vector, preferably replication competent, that comprises E3 region (or a portion of E3) under transcriptional control of a target cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a native adenoviral TRE, and the vector further

comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a target cell-specific TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE.

5   *Transgenes under transcriptional control of a target cell-specific TRE*

Various other replication-competent adenovirus vectors can be made according to the present invention in which, in addition to having a single or multiple adenovirus gene(s) under control of a target cell-specific TRE, a transgene(s) is/are also under control of a target cell-specific TRE and optionally under translational control of an IRES. Transgenes

10   include, but are not limited to, therapeutic transgenes and reporter genes.

Reporter genes

For example, a target cell-specific TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A or E1B, and · this construct may further comprise a second co-transcribed gene under translational

15   control of an IRES. The second gene may be a reporter gene. The reporter gene can . encode a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the *lacZ* gene), luciferase, alkaline phosphatase, a green fluorescent protein, and horse radish peroxidase. For detection of a putative cancer cell(s) in a biological sample, the biological sample may be treated with modified

20   adenoviruses in which a reporter gene (e.g., luciferase) is under control of a target cell-specific TRE. The target cell-specific TRE will be transcriptionally active in cells that allow the target cell-specific TRE to function, and luciferase will be produced. This production will allow detection of target cells, including cancer cells in, for example, a human host or a biological sample. Alternatively, an adenovirus can be constructed in

25   which a gene encoding a product conditionally required for survival (e.g., an antibiotic resistance marker) is under transcriptional control of a target cell-specific TRE. When this adenovirus is introduced into a biological sample, the target cells will become antibiotic resistant. An antibiotic can then be introduced into the medium to kill the non-cancerous cells.

30   Therapeutic transgenes

Transgenes also include genes which may confer a therapeutic effect, such as enhancing cytotoxicity so as to eliminate unwanted target cells. In this way, various genetic capabilities may be introduced into target cells, particularly cancer cells. For

example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the cancerous target cell. This could be accomplished by coupling the target cell-specific cytotoxic activity with cell-specific expression of, for example, HSV-tk

5   and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or

10 abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507; Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other

15 capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly; genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. awsin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest

20 include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN-α, -β, -χ, TNF-α, -β, TGF-α, -β, NGF, and the like. The positive effector genes could be used in an earlier phase, followed by cytotoxic activity due to replication.

Host cells

25   The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Host systems are known in the art and need not be described in detail herein. Prokaryotic

30 host cells include bacterial cells, for example, *E. coli, B. subtilis,* and mycobacteria. Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Examples of fungi (including yeast) host cells are *S. cerevisiae, Kluyveromyces lactis (K. lactis),* species of *Candida* including *C. albicans* and *C. glabrata,*

Aspergillus nidulans, Schizosaccharomyces pombe (S. pombe), Pichia pastoris, and
Yarrowia lipolytica. Examples of mammalian cells are cultured human target cells (HUC),
KU-1, MYP3 (a non-tumorigenic rat target cell line), 804G (rat bladder carcinoma cell
line), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. COS cells, mouse L
5   cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK)
cells, and African green monkey cells. *Xenopus laevis* oocytes, or other cells of amphibian
origin, may also be used.

Compositions and kits

The present invention also includes compositions, including pharmaceutical
10   compositions, containing the adenoviral vectors described herein. Such compositions are
useful for administration *in vivo*, for example, when measuring the degree of transduction
and/or effectiveness of cell killing in an individual. Compositions can comprise an
adenoviral vector(s) of the invention and a suitable solvent, such as a physiologically
acceptable buffer. These are well known in the art. In other embodiments, these
15   compositions further comprise a pharmaceutically acceptable excipient. These
compositions, which can comprise an effective amount of an adenoviral vector of this
invention in a pharmaceutically acceptable excipient, are suitable for systemic or local
administration to individuals in unit dosage forms, sterile parenteral solutions or
suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or
20   water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug
delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*,
19th Edition, Mack Publishing (1995). Compositions also include lyophilized and/or
reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as
adenovirus) of the invention.

25   The present invention also encompasses kits containing an adenoviral vector(s) of
this invention. These kits can be used for diagnostic and/or monitoring purposes,
preferably monitoring. Procedures using these kits can be performed by clinical
laboratories, experimental laboratories, medical practitioners, or private individuals. Kits
embodied by this invention allow someone to detect the presence of bladder cancer cells in
30   a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable
packaging. The kit may optionally provide additional components that are useful in the

procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Preparation of the adenovirus vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant

5   techniques that are standard in the art. Generally, a target cell-specific TRE is inserted 5' to the adenoviral gene of interest, preferably an adenoviral replication gene, more preferably one or more early replication genes (although late gene(s) can be used). A target cell-specific TRE can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient

10   restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for a target cell-specific TRE. Accordingly, convenient restriction sites for annealing (i.e., inserting) a target cell-specific TRE can be engineered onto the 5' and 3' ends of a UP-TRE using standard recombinant methods, such as PCR.

15   Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art, such as chemical synthesis, recombinant methods and/or obtained from biological sources.

Adenoviral vectors containing all replication-essential elements, with the desired elements (e.g., E1A) under control of a target cell-specific TRE, are conveniently prepared

20   by homologous recombination or *in vitro* ligation of two plasmids, one providing the left-hand portion of adenovirus and the other plasmid providing the right-hand region, one or more of which contains at least one adenovirus gene under control of a target cell-specific TRE. If homologous recombination is used, the two plasmids should share at least about 500 bp of sequence overlap. Each plasmid, as desired, may be independently manipulated,

25   followed by cotransfection in a competent host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from a target cell-specific TRE for propagation of the adenovirus. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as cationic liposomes. Alternatively, *in vitro* ligation of the right and

30   left-hand portions of the adenovirus genome can also be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Bridge et al. (1989) *J. Virol.* 63: 631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHG10 (Bett et al. (1994); Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The deletion in E3 provides

5   room in the virus to insert a 3 kb target cell-specific TRE without deleting the endogenous enhancer/promoter. The gene for E3 is located on the opposite strand from E4 (r-strand). pBHG11 provides an even larger E3 deletion (an additional 0.3 kb is deleted). Bett et al. (1994). Alternatively, the use of pBHGE3 (Microbix Biosystems, Inc.) provides the right hand end of Ad5, with a full-length of E3.

10   For manipulation of the early genes, the transcription start site of Ad5 E1A is at 498 and the ATG start site of the E1A coding segment is at 560 in the virus genome. This region can be used for insertion of a target cell-specific TRE. A restriction site may be introduced by employing polymerase chain reaction (PCR), where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid

15   carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a nucleotide sequence change resulting in a unique restriction site, one can provide for insertion of target cell-specific TRE at that site.

20   A similar strategy may also be used for insertion of a target cell-specific TRE element to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Sp1 and a TATA box. This region extends from Ad5 nt 1636 to 1701. By insertion of a target cell-specific TRE in this region, one can provide for cell-specific transcription of the E1B gene. By employing the left-hand region modified with the cell-

25   specific response element regulating E1A, as the template for introducing a target cell-specific TRE to regulate E1B, the resulting adenovirus vector will be dependent upon the cell-specific transcription factors for expression of both E1A and E1B. In additional embodiments, the 19-kDa region of E1B is deleted.

Similarly, a target cell-specific TRE can be inserted upstream of the E2 gene to

30   make its expression cell-specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of

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the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Immunol.* (1995) 199(part 3):177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2

5 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a target

10 cell-specific TRE having SpeI ends into the SpeI site in the 1-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow target cell-restricted expression of E2 transcripts.

For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at about nt 35605, the TATA box at about nt 35631

15 and the first AUG/CUG of ORF I is at about nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a UP-TRE may be introduced upstream from the transcription start site. For the construction of full-length adenovirus with a target cell-specific TRE inserted in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg et al. (1983) *Proc.*

20 *Natl. Acad. Sci.* 80:5383–5386) which provide E4 proteins *in trans* to complement defects in synthesis of these proteins.

Adenoviral constructs containing an E3 region can be generated wherein homologous recombination between an E3-containing adenoviral plasmid, for example, BHGE3 (Microbix Biosystems Inc., Toronto) and a non-E3-containing adenoviral plasmid,

25 is carried out.

Alternatively, an adenoviral vector comprising an E3 region can be introduced into cells, for example 293 cells, along with an adenoviral construct or an adenoviral plasmid construct, where they can undergo homologous recombination to yield adenovirus containing an E3 region. In this case, the E3-containing adenoviral vector and the

30 adenoviral construct or plasmid construct contain complementary regions of adenovirus, for example, one contains the left-hand and the other contains the right-hand region, with sufficient sequence overlap as to allow homologous recombination.

Alternatively, an E3-containing adenoviral vector of the invention can be constructed using other conventional methods including standard recombinant methods (e.g., using restriction nucleases and/or PCR), chemical synthesis, or a combination of any of these. Further, deletions of portions of the E3 region can be created using standard

5   techniques of molecular biology.

Insertion of an IRES into a vector is accomplished by methods and techniques that are known in the art and described herein *supra*, including but not limited to, restriction enzyme digestion, ligation, and PCR. A DNA copy of an IRES can be obtained by chemical synthesis, or by making a cDNA copy of, for example, a picornavirus IRES. *See,*

10   for example, Duke et al. (1995) *J.Vvirol.* 66(3):1602-9) for a description of the EMCV IRES and Huez et al. (1998), *Mol. Cell. Biol.* 18(11):6178-90) for a description of the VEGF IRES. The internal translation initiation sequence is inserted into a vector genome at a site such that it lies upstream of a 5'-distal coding region in a multicistronic mRNA. For example, in a preferred embodiment of an adenovirus vector in which production of a

15   bicistronic E1A-E1B mRNA is under the control of a target cell-specific TRE, the E1B promoter is deleted or inactivated, and an IRES sequence is placed between E1A and E1B. IRES sequences of cardioviruses and certain aphthoviruses contain an AUG codon at the 3' end of the IRES that serves as both a ribosome entry site and as a translation initiation site. Accordingly, this type of IRES is introduced into a vector so as to replace the translation

20   initiation codon of the protein whose translation it regulates. However, in an IRES of the entero/rhinovirus class, the AUG at the 3' end of the IRES is used for ribosome entry only, and translation is initiated at the next downstream AUG codon. Accordingly, if an entero/rhinovirus IRES is used in a vector for translational regulation of a downstream coding region, the AUG (or other translation initiation codon) of the downstream gene is

25   retained in the vector construct.

Methods of packaging polynucleotides into adenovirus particles are known in the art and are also described in co-owned PCT PCT/US98/04080.

Delivery of Adenovirus vectors

The adenoviral vectors can be used in a variety of forms, including, but not limited

30   to, naked polynucleotide (usually DNA) constructs. Adenoviral vectors can, alternatively, comprise polynucleotide constructs that are complexed with agents to facilitate entry into cells, such as cationic liposomes or other cationic compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more

immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents (such as PEG) to enhance or dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMA™, DOTAP™, and polyamines.

5   If an adenoviral vector comprising an adenovirus polynucleotide is packaged into a whole adenovirus (including the capsid), the adenovirus itself may also be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virol.* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type

10 and/or reduced affinity for non-target cell types, such subgenus(or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art, such as calcium phosphate precipitation, electroporation, direct injection, and

15 intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used in packaged adenoviruses, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to about 10^{14}. The

20 multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μg to about 1000 μg of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple, simultaneous injections.

25 If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined

30 empirically.

Methods using the adenovirus vectors of the invention

The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

5   In one embodiment, methods are provided for conferring selective cytotoxicity in cells that allow a target cell-specific TRE to function, preferably target cells, comprising contacting such cells with an adenovirus vector described herein. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ^{3}H-thymidine incorporation, and/or lysis.

10   In another embodiment, methods are provided for propagating an adenovirus specific for cells which allow a target cell-specific TRE to function, preferably target cells, preferably cancer cells. These methods entail combining an adenovirus vector with the cells, whereby said adenovirus is propagated.

         Another embodiment provides methods for killing cells that allow a target cell-

15   specific TRE to function in a mixture of cells, comprising combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally a mixture of normal cells and cancerous cells that allow a target cell-specific TRE to function, and can be an *in vivo* mixture or *in vitro* mixture.

         The invention also includes methods for detecting cells which allow a target cell-

20   specific TRE to function, such as cancer cells, in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. In one method, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. Alternatively, the sample can be contacted with an

25   adenovirus in which a reporter gene is under control of a target cell-specific TRE. When such an adenovirus is introduced into a biological sample, expression of the reporter gene indicates the presence of cells that allow a target cell-specific TRE to function. Alternatively, an adenovirus can be constructed in which a gene conditionally required for cell survival is placed under control of a target cell-specific TRE. This gene may encode,

30   for example, antibiotic resistance. Later the biological sample is treated with an antibiotic. The presence of surviving cells expressing antibiotic resistance indicates the presence of cells capable of target cell-specific TRE function. A suitable biological sample is one in which cells that allow a target cell-specific TRE to function, such as cancer cells, may be or

65

are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells that allow a target cell-specific TRE to function, such as carcinoma cells, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay

5   conditions, such as selective enrichment, and/or solubilization. In these methods, cells that allow a target cell-specific TRE to function can be detected using *in vitro* assays that detect adenoviral proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yield) and plaque assays (which measure infectious particles per cell). Propagation can also be detected by

10   measuring specific adenoviral DNA replication, which are also standard assays.

The invention also provides methods of modifying the genotype of a target cell, comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention further provides methods of suppressing tumor cell growth,

15   preferably a tumor cell that allows a target cell-specific TRE to function, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed

20   below), radiation and/or antibodies.

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the present invention, wherein the adenoviral vector is selectively cytotoxic toward cells that allow a target cell-specific TRE to function. Tumor cell markers include, but are not

25   limited to, CK-20. Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to, immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample. For

30   these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. Treatment using an adenoviral vector(s) is indicated in individuals with cancer as described above. Also indicated are individuals who are considered to be at risk for developing cancer (including

5   single cells), such as those who have had disease which has been resected and those who have had a family history of cancer. Determination of suitability of administering adenoviral vector(s) of the invention will depend, inter alia, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) in a

10   pharmaceutically acceptable excipient is administered. Pharmaceutical compositions are described above. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The amount of adenoviral vector(s) to be administered will depend on several

15   factors, such as route of administration, the condition of the individual, the degree of aggressiveness of the disease, the particular target cell-specific TRE employed, and the particular vector construct (i.e., which adenovirus gene(s) is under target cell-specific TRE control) as well as whether the adenoviral vector is used in conjunction with other treatment modalities.

20   If administered as a packaged adenovirus, from about 10^4 to about 10^{14}, preferably from about 10^4 to about 10^{12}, more preferably from about 10^4 to about 10^{10}. If administered as a polynucleotide construct (i.e., not packaged as a virus), about 0.01 µg to about 100 µg can be administered, preferably 0.1 µg to about 500 µg, more preferably about 0.5 µg to about 200 µg. More than one adenoviral vector can be administered, either

25   simultaneously or sequentially. Administrations are typically given periodically, while monitoring any response. Administration can be given, for example, intratumorally, intravenously or intraperitoneally.

The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective.

30   Examples of chemotherapeutics which are suitable for suppressing bladder tumor growth are BGC (bacillus Calmett-Guerin); mitomycin-C; cisplatin; thiotepa; doxorubicin; methotrexate; paclitaxel (TAXOL™); ifosfamide; gallium nitrate; gemcitabine;

carboplatin; cyclosphasphamid; vinblastine; vincristin; fluorouracil; etoposide; bleomycin. Examples of combination therapies include (CISCA (cyclophosphamide, doxorubicin, and cisplatin); CMV (cisplatin, methotrexate, vinblastine); MVMJ (methodtrextate, vinblastine, mitoxantrone, carboplain); CAP (cyclophosphamide, doxorubicin, cisplatin); MVAC

5   (methotrexate, vinblastine, doxorubicin, cisplatin). Radiation may also be combined with chemotherapeutic agent(s), for example, radiation with cisplatin. Administration of the chemotherapeutic agents is generally intravesical (directly into the bladder) or intravenous.

The following examples are provided to illustrate but not limit the invention.

10   EXAMPLES

Example 1: Construction of a replication-competent adenovirus vector comprising an AFP-TRE and an EMCV IRES

The encephalomyocarditis virus (ECMV) IRES as depicted in Table 1 was introduced between the E1A and E1B regions of a replication-competent adenovirus vector

15   specific for cells expressing AFP as follows. Table 1 shows the 519 base pair IRES segment which was PCR amplified from Novagen's pCITE vector by primers A/B as listed in Table 4. A 98 base pair deletion in the E1A promoter region was created in PXC.1, a plasmid which contains the left-most 16 mu of Ad5. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5, from Adenovirus 5 nt 22 to

20   5790 including the inverted terminal repeat, the packaging sequence, and the E1a and E1b genes in vector pBR322. pBHG10 (Bett. et al. (1994) Proc. Natl. Acad. Sci. USA 91:8802-8806; Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The resultant plasmid, CP306 (PCT/US98/16312), was used as the backbone in overlap PCR to generate CP624. To place a Sal1 site between E1a and E1b,

25   primers C/D, E/F (Table 4) were used to amplify CP306, plasmid derived from pXC.1 and lacking the E1a promoter. After first round PCR using CP306 as template and primers C/D, E/F, the resultant two DNA fragments were mixed together for another round of overlapping PCR with primers C/F. The overlap PCR product was cloned by blunt end ligation to vector. The resultant plasmid, CP624 (Table 5), contains 100 bp deletion in

30   E1a/E1b intergenic region and introduces Sal1 site into the junction. On this plasmid, the endogenous E1a promoter is deleted, and the E1a polyadenylation signal and the E1b promoter are replaced by the Sal1 site. Next, the Sal1 fragment of CP625 was cloned into the Sal1 site in CP624 to generate CP627 (Table 5). CP627 has an EMCV IRES connecting

adenovirus essential genes E1a and E1b. In CP627, a series of different tumor-specific promoters can be placed at the *PinAl* site in front of E1a to achieve transcriptional control on E1 expression.

Table 4

Primer	Sequence	Note
A.	5'-GACGTCGACTAATTCCGGTTATTTTCCA	For PCR EMCV IRES, *GTCGAC* is a Sal I site.
B.	5'-GACGTCGACATCGTGTTTTTCAAAGGAA	For PCR EMCV IRES, *GTCGAC* is a Sal I site.
C.	5'-CCTGAGACGCCCGACATCACCTGTG	Ad5 sequence to 1314 to 1338.
D.	5'-<u>GTCGACCATTCAGCA</u>AACAAAGGCGTTAAC	Antisense of Ad5 sequence 1572 to 1586. *GTCGAC* is a Sal I site. Underline region overlaps with E.
E.	5'-<u>TGCTGAATGGTCGACA</u>TGGAGGCTTGGGAG	Ad5 sequence 1714 to 1728. *GTCGAC* is a Sal I site. Underline region overlaps with D.
F.	5'-CACAAACCGCTCTCCACAGATGCATG	Antisense of Ad5 sequence 2070 to 2094.

5   For generating a liver cancer-specific virus, an about 0.8kb AFP promoter fragment as shown in Table 3 was placed into the *PinAl* site of CP627 thereby yielding plasmid CP686. Full-length viral genomes were obtained by recombination between CP686 and a plasmid containing a right arm of an adenovirus genome. The right arms used in virus

10   recombination were pBHGE3 (Microbix Biosystems Inc.), containing an intact E3 region, and pBHG11 or pBHG10 (Bett et al. (1994) containing a deletion in the E3 region.

The virus obtained by recombination of CP686 with a right arm containing an intact E3 region was named CV890. The virus obtained by recombination of CP686 with a right arm containing a deleted E3 region (pBHG 10) was named CV840. The structure of all

15   viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

Therefore, adenovirus vector designated CV890 comprises 0.8 kb AFP promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B a deletion of the E1B promoter and an intact E3 region. Adenovirus vector CV840 comprises AFP promoter, E1A, a

20   deletion of the E1A promoter, EMCV IRES, E1B, a deletion of the E1B promoter and a deleted E3 region.

Table 5

Plasmid designation	Brief description
CP306	An E1A promoter deleted plasmid derived from pXC.1

CP624	Overlap PCR product from CP306 to generate 100 bp deletion and introduce a SalI site at E1A and E1B junction; E1A and E1B promoter deleted in E1A/E1B intergenic region.
CP625	EMCV IRES element ligated to PCR-blunt vector (Invitrogen pCR® blunt vector).
CP627	IRES element derived from CP625 by SalI digestion and ligated to CP624 SalI site placing IRES upstream from E1B.
CP628	Probasin promoter derived from CP251 by PinAI digestion and cloned into PinAI site on CP627.
CP629	HCMV IE promoter amplified from pCMV beta (Clontech) with PinAI at 5' and 3' ends ligated into CP627 PinAI site.
CP630	A 163 bp long VEGF IRES fragment (Table 1) cloned into the SalI site on CP628.
CP686	AFP promoter from CP219 digested with PinAI and cloned into PinAI site on CP627.

Example 2: Construction of a replication-competent adenovirus vector with a probasin TRE and an EMCV IRES

The probasin promoter as shown in Table 3 was inserted at the PinAI site of
plasmid CP627 (see Example 1) to generate CP628, which contains a probasin promoter
upstream of E1A and an EMCV IRES between E1A and E1B. Full-length viral genomes
were obtained by recombination between CP628 and a plasmid containing a right arm of an
adenovirus genome. The right arms used in virus recombination were pBHGE3, containing
an intact E3 region, and pBHG11 or pBHG10 containing a deletion in the E3 region. The
structure of all viral genomes was confirmed by conducting PCR amplifications that were
diagnostic for the corresponding specific regions.

Therefore, adenovirus designated CV 834 comprises probasin promoter, E1A, a
deletion of the E1A promoter, EMCV IRES, E1B, a deletion of the E1B endogenous
promoter and a deleted E3 region.

Example 3: Construction of a replication-competent adenovirus vector with a hCMV-TRE and an EMCV IRES

The hCMV immediate early gene (IE) promoter from plasmid CP629, originally
derived from pCMVBeta (Clonetech, Palo Alto) was inserted at the PinAI site of plasmid
CP627 (see Example 1) to generate CP629, containing a CMV IE promoter upstream of
E1A and an IRES between E1A and E1B. Full-length viral genomes were obtained by
recombination between CP629 and a plasmid containing a right arm of an adenovirus

genome. The right arms used in virus recombination were pBHGE3, containing an intact

E3 region, and pBHG11 or pBHG10 containing a deletion in the E3 region. The structure

of all viral genomes was confirmed by conducting PCR amplifications that were diagnostic

for the corresponding specific regions.

5   Therefore, adenovirus vector designated CV835 comprises hCMV-IE promoter,

E1A, a deletion of the E1A promoter, EMCV IRES, E1B a deletion in the E1B endogenous

promoter and a deleted E3 region. CV835 lacks the hCMV enhancer and is therefore not

tissue specific. By adding the hCMV IE enhancer sequence to CV835, the vector is made

tissue specific.

10

**Example 4: Replication of IRES-containing adenovirus vectors with different
TREs controlling E1 expression**

The viral replication of adenovirus vectors comprising the probasin promoter

(CV836 and CV834) generally considered a weak promoter, and the human

15   cytomegalovirus immediate early gene (HCMV-IE) promoter (CV837 and CV835),

generally considered a strong promoter, were characterized in the virus yield assay.

Probasin promoter containing adenovirus vectors (see PCT/US98/04132), CV836

and CV834, and HCMV-IE promoter containing adenovirus vectors, CV837 and CV835,

were tested against a panel of cell lines for viral replication (indicative of lethality) and

20   specificity. Cell lines 293 (the producer line), LNCap and HepG2 were plated at 0.5×10^6

per well in 6 well tissue culture plates, incubated for 24 hours at 37° C, then infected with

CV836, CV834 or CV837 and CV835 at a multiplicity of infection (MOI) of 2 plaque

forming units per cell (PFU/cell) for 4 hours at 37° C. At the end of the infection period,

the medium was replaced and the cells were incubated at 37° C for a further 72 hours

25   before harvesting for a viral yield assay as described in Yu et al. (1999) *Cancer

Res.* 59:1498-1504. The results are shown in Fig. 3.

The data demonstrate that the presence of an IRES element in the intergenic region

between E1A and E1B does not significantly affect viral replication, as compared to control

viruses lacking an IRES, such as a wild-type AD5 with a deletion in the E3 region. In

30   CV834, the loss of tissue cytotoxicity could be caused by the weakness of the probasin

promoter in the virus structure.

Example 5:   Comparison of dual TRE vectors with single TRE/IRES-containing vectors

Two liver cancer-specific adenovirus vectors, CV790 and CV733 (also designated CN790 and CN733, respectively), were generated and characterized. See

5   PCT/US98/04084. These viruses contain two AFP TREs, one upstream of E1A and one upstream of E1B. They differ in that CV790 contains an intact E3 region, while the E3 region is deleted in CV733. Replication of these two viruses was compared with that of the newly generated IRES-containing viruses, CV890 and CV840 (*see* Example 1).

Virus replication was compared, in different cell types, using a virus yield assay as

10   described in Example 4. Cells were infected with each type of virus and, 72 hrs after infection, virus yield was determined by a plaque assay. Figs. 4A and 4B show viral yield for different viruses in different cell types. The results indicate that vectors containing an IRES between E1A and E1B (CV890 and CV840), in which E1B translation is regulated by the IRES, replicate to similar extents as normal adenovirus and viruses with dual AFP

15   TREs, in AFP-producing cells such as 293 cells and hepatoma cells. In SK-Hep-1 (liver cells), PA-1 (ovarian carcinoma) and LNCaP cells (prostate cells) the IRES-containing viruses do not replicate as well as dual TRE or wild-type adenoviruses, indicating that the IRES-containing viruses have higher specificity for hepatoma cells. Based on these results, it is concluded that IRES-containing vectors have unaltered replication levels, but are more

20   stable and have better target cell specificity, compared to dual-TRE vectors.

Example 6:   Uroplakin adenoviral constructs containing an EMCV IRES

A number of E3-containing viral constructs were prepared which contained uroplakin II sequences (mouse and/or human) as well as an EMCV internal ribosome entry

25   site (IRES). The viral constructs are summarized in Table 6. All of these vectors lacked an E1A promoter and retained the E1A enhancer.

The 519 base pair EMCV IRES segment was PCR amplified from Novagen's pCITE vector by primers A/B:

primer A: 5'-GACGTCGACTAATTCCGGTTATTTTCCA

30   primer B 5'-GACGTCGACATCGTGTTTTTCAAAGGAA (*GTCGAC* is a SalI site).

The EMCV IRES element was ligated to PCR blunt vector (Invitrogen pCR® blunt vector).

CP1066

5   The 1.9kb-(-1885 to +1) fragment of mouse UPII from CP620 was digested with AflIII (blunted) and HindIII and inserted into pGL3-Basic from CP620 which had been digested with XhoI (blunted) and HindIII to generate CP1066.

CP1086

10   The 1.9kb mouse UPII insert was digested with PinAI and ligated with CP269 (CMV driving E1A and IRES driving E1B with the deletions of E1A/E1B endogenous promoter) which was similarly cut by PinAI.

CP1087

15   The 1kb (-1128 to +1) human UPII was digested with PinAI from CP665 and inserted into CP629 which had been cut by PinAI and purified (to elute CMV).

CP1088

The 2.2kb (-2225 to +1) human UPII was amplified from CP657 with primer
20   127.2.1 (5'-AGGACCGGTCACTATAGGGCACGCGTGGT-3') PLUS 127.2.2 (5'-AGGACCGGTGGGATGCTGGGCTGGGAGGTGG-3') and digested with PinAI and ligated with CP629 cut with PinAI.

CP627 is an Ad5 plasmid with an internal ribosome entry site (IRES) from encephelomycarditis virus (EMCV) at the junction of E1A and E1B. First, CP306 (Yu et
25   al., 1999) was amplified with primer pairs 96.74.3/96.74.6 and 96.74.4/96.74.5.

The two PCR products were mixed and amplified with primer pairs 96.74.3 and 96.74.5. The resultant PCR product contains a 100bp deletion in E1A-E1B intergenic region and a new SalI site at the junction. EMCV IRES fragment was amplified from pCITE-3a(+) (Novagen) using primers 96.74.1 and 96.74.2. The SalI fragment containing
30   IRES was placed into SalI site to generate CP627 with the bicistronic E1A-IRES-E1B

cassette. CP629 is a plasmid with CMV promoter amplified from pCMVbeta (Clontech) with primer 99.120.1 and 99.120.2 and cloned into PinAI site of CP627.

CP657 is a plasmid with 2.2kb 5' flanking region of human UP II gene in pGL3-Basic (Promega). The 2.2kb hUPII was amplified by PCR from GenomeWalker product
5   with primer 100.113.1 and 100.113.2 and TA-cloned into pGEM-T to generate CP655.

The 2.2kb insert digested from SacII (blunt-ended) and KpnI was cloned into pGL3-Basic at HindIII (blunted) and KpnI to create CP657.

CP1089

10   The 1kb (-965 to +1) mouse UPII was digested by PinAI from CP263 and inserted into CN422 (PSE driving E1A and GKE driving E1B with the deletions of E1A/E1B endogenous promoter) cut by PinAI and purified and further digested with EagI and ligated with 1kb (-1128 to +1) human UPII cut from CP669 with EagI.

CP1129

15   The 1.8kb hUPII fragment with PinAI site was amplified from CP657 with primer 127.50.1 and 127.2.2 and cloned into PinAI site of CP629.

CP1131

CP686 was constructed by replacing the CMV promoter in CP629 with an AFP fragment from CP219. A 1.4kb DNA fragment was released from CP686 by digesting it
20   with BssHII, filling with Klenow, then digesting with BglII. This DNA fragment was then cloned into a similarly cut CP686 to generate CP1199. In CP1199, most of the E1B 19-KDa region was deleted. The 1.8kb hUPII fragment with PinAI site was amplified from CP657 by PCR with primer 127.50.1 and 127.2.2 and inserted into similarly digested CP1199 to create CP1131.

25   The plasmids above were all co-transfected with pBHGE3 to generate CV874 (from CP1086), CV875 (from CP1087), CV876 (from 1088) and CV877 (from CP1089), CV882 (from CP1129) and CV884 (from CP1131). CP1088, CP1129 and CP1131 were cotransfected with pBHGE3 for construction of CV876, CV892 and CV884, respectively by lipofectAMINE (Gibco/BRL) for 11-14 days. pBHGE3 was purchased from
30   Microbix, Inc., and was described previously. The cells were lysed by three freeze-thaw

cycles and plaqued on 293 cells for a week. The single plaques were picked and amplified by infection in 293 cells for 3-5 days. The viral DNAs were isolated from the lysates and the constructs were confirmed by PCR with primer 31.166.1/ 51.176 for CV876 and primer 127.50.1/51.176 for CV882 and CV884 at E1 region and primer 32.32.1/2 for all three viruses at E3 region.

TABLE 6

Name	Vector	Ad 5 Vector	E1A TRE	E1B TRE	E3
CV874	CP1086	pBHGE3	1.9 kb mUPII	IRES	intact
CV875	CP1087	pBHGE3	1.0 kb hUPII	IRES	intact
CV876	CP1088	pBHGE3	2.2 kb hUPII	IRES	intact
CV877	CP1089	pBHGE3	1.0 kb mUPII	1.0 kb hUPII (E1B promoter deleted)	intact
CV882	CP1129	pBHGE3	1.8 kb hUPII	IRES	intact
CV884	CP1131	pBHGE3	1.8 kb hUPii	IRES (E1B 19-kDa deleted)	intact

Viruses are tested and characterized as described above.

Primer sequences:

96.74.1	GACGTCGACATCGTGTTTTTCAAAGGAA
96.74.2	GACGTCGACTAATTCCGGTTATTTTCCA
96.74.3	CCTGAGACGCCCGACATCACCTGTG
96.74.4	TGCTGAATGGTCGACATGGAGGCTTGGGAG
96.74.5	CACAACCGCTCTCCACAGATGCATG
96.74.6	GTCGACCATTCAGCAAACAAAGGCGTTAAC
100.113.1	AGGGGTACCCACTATAGGGCACGCGTGGT
100.113.2	ACCCAAGCTTGGGATGCTGGGCTGGGAGGTGG
127.2.2	AGGACCGGTGGGATGCTGGGCTGGGAGGTGG
127.50.1	AGGACCGGTCAGGCTTCACCCCAGACCCAC
31.166.1	TGCGCCGGTGTACACAGGAAGTGA
32.32.1	GAGTTTGTGCCATCGGTCTAC
32.32.2	AATCAATCCTTAGTCCTCCTG
51.176	GCAGAAAAATCTTCCAAACACTCCC
99.120.1	ACGTACACCGGTCGTTACATAACTTAC
99.120.2	CTAGCAACCGGTCGGTTCACTAAACG

Example 7: Construction of a replication-competent adenovirus vector with a tyrosinase TRE and EMCV IRES

CP621 is a plasmid containing a human tyrosinase enhancer and promoter elements in a PinAl fragment. This fragment is ligated to the PinAl site on CP627 to generate

5   CP1078. CP1078 is combined with pBHGE3 to generate a new melanoma specific virus, CV859. Table 3 depicts the polynucleotide sequence of the PinAl fragment which contains a tyrosinase promoter and enhancer.

Example 8: Construction of a replication-competent adenovirus vector with a

10   **probasin-TRE and a VEGF IRES**

Using a strategy similar to that described in Example 1, the IRES fragment from the mouse vascular endothelial growth factor (VEGF) gene is amplified and cloned into CP628 at the SalI site. Table 1 depicts the IRES fragment obtainable from vascular endothelial growth factor (VEGF) mRNA. In order to clone this fragment into the E1a/E1b intergenic

15   region, two pieces of long oligonucleotide are synthesized. The sense oligonucleotide is shown in the Table, whereas the second piece is the corresponding antisense one. After annealing the two together to create a duplex, the duplex is subjected to SalI digestion and the resulting fragment is cloned into the SalI site on CP628. The resulting plasmid, CP630, has a probasin promoter in front of E1a and an VEGF IRES element in front of E1b. This

20   plasmid is used to construct a prostate cancer-specific virus comprising the VEGF IRES element.

Example 9: Construction of a replication-competent adenovirus vector with an AFP-TRE and a VEGF IRES

25   Using a strategy similar to Example 1, a PinAI fragment which contains AFP TRE can be obtained. This AFP TRE is cloned into the PinA1 site in front of E1A on CP628 yielding plasmid CP1077. This plasmid has the AFP TRE for E1 transcriptional control and the VEGF IRES element before E1b. CP1077 can be recombined with pBHGE3 to generate a liver-specific adenovirus, designated as CV858.

30

Example 10: Construction of a replication-competent adenovirus vector with a hKLK2-TRE and a EMCV IRES

Using a strategy similar to Example 1, the TRE fragment from human glandular kallikrein II as shown in Table 3 was cloned into the PinAI site in CP627. The resultant
5   plasmid, CP1079, is cotransfected with pBHGE3 to create CV860.

Example 11: Treatment of Hep3B Tumor Xenografts with Replication-Competent Hepatoma Specific CV790 and Doxorubicin and Hepatoma Specific CV890 and Doxorubicin

10   CV790 is an AFP producing hepatocellular carcinoma specific adenovirus, with EIA and E1B under the control of an identical AFP promoter and enhancer (822 base pair promoter shown in Table 3) with an E3 region. The CV890 adenovirus construct is also a hepatoma or liver-specific adenoviral mutant with the E1A and E1B genes under transcriptional control of 822bp AFP promoter (827 bp including nucleotides for restriction
15   site), wherein E1B is under translational control of EMCV IRES and having an intact E3 region. The structure of CV890 therefore reads as AFP/E1A, IRES/E1B, E2, E3, E4. *In vivo* studies of the efficacy of combinations of CV790 and doxorubicin and CV890 and doxorubicin were performed according to the protocols described in detail in Example 4, with minor alterations which are described below.

20   Xenografts in the study of CV790 and CV890 combined with chemotherapeutic agents utilized liver carcinoma Hep3B cells. Virus, CV790 or CV890, was administered by a single intravenous injection of 1×10^{11} particles through the tail veins of the nude mice. One day after virus delivery, a single dose of doxorubicin was given to each animal by i.p. injection. The doxorubicin dose was 10 mg/kg for both doxorubicin alone and doxorubicin
25   combined with virus treatments. Tumor volume was measured once a week for six weeks. Tumors were measured weekly in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width $(mm)^2$]/2.

Figure 8 depicts the anti-tumor activity of CV890 containing an IRES as compared to CV 790 containing dual TREs. As Figure 8 demonstrates, relative tumor volume was
30   less with administration of CV890 than administration of CV790. Furthermore, both CV790/doxorubicin and CV890/doxorubicin treatment of the hepatoma showed synergistic results. Four days after treatment with either CV790/doxorubicin or CV890/doxorubicin the relative tumor volume was less than 10%. Unlike mice treated with either virus alone

or doxorubicin alone, after day 4, the relative tumor volume did not increase for either the either CV790/doxorubicin or CV890/doxorubicin treated mice. At day 6 in the control mice, the relative tumor volume was approximately 1000% in the CV790 study and approximately 600% in the CV890 study. The relative tumor volumes of mice treated with 5   virus alone were 250% (CV790) and 520% (CV890) while the relative tumor volumes for mice treated with doxorubicin alone were 450% with 280% in the CV790 study and 500% in the CV890 study.

        Example 12: *In vitro* characterization of melanocyte-specific TRE-containing
10   **adenoviral constructs**

        An especially useful objective in the development of melanocyte cell-specific adenoviral vectors is to treat patients with melanoma. Methods are described below for measuring the activity of a melanocyte-specific TRE and thus for determining whether a given cell allows a melanocyte-specific TRE to function.

15   *Cells and Culture Methods*

        Host cells such as, HepG2 (liver); Lovo (colon); LNCaP (prostate); PMEL (melanoma); SKMel (melanoma); G361 (melanoma) and MeWo cells are obtained at passage 9 from the American Type Culture Collection (Rockville, MD). MeWo cells are maintained in RPMI 1640 medium (RPMI) supplemented with 10% fetal bovine serum 20   (FBS; Intergen Corp.), 100 units/mL of penicillin, and 100 units/mL streptomycin. MeWo cells being assayed for luciferase expression are maintained in 10% strip-serum (charcoal/dextran treated fetal bovine serum to remove T3, T4, and steroids; Gemini Bioproduct, Inc., Calabasas, CA) RPMI.

        Transfections of MeWo Cells

25   For transfections, MeWo cells are plated out at a cell density of 5 x 10^5 cells per 6-cm culture dish (Falcon, NJ) in complete RPMI. DNAs are introduced into MeWo cells after being complexed with a 1:1 molar lipid mixture of N-[1-(2,3-dioleyloxy)propyl-*N,N,N*-trimethylammonium chloride (DOTAP™; Avanti Polar Lipids, AL) and dioleoyl-phosphatidylethanolamine (DOPE™; Avanti Polar Lipids, AL); DNA/lipid complexes are 30   prepared in serum-free RPMI at a 2:1 molar ratio. Typically, 8 μg (24.2 nmole) of DNA is diluted into 200 μL of incomplete RPMI and added dropwise to 50 nmole of transfecting, lipids in 200 μL of RPMI with gentle vortexing to insure homogenous mixing of

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components. The DNA/lipid complexes are allowed to anneal at room temperature for 15 minutes prior to their addition to MeWo cells. Medium is removed from MeWo cells and replaced with 1 mL of serum-free RPMI followed by the dropwise addition of DNA/lipid complexes. Cells are incubated with complexes for 4-5 hours at 37°C, 5% CO_2. Medium

5   was removed and cells washed once with PBS. The cells were then trypsinized and resuspended in 10% strip-serum RPMI (phenol red free). Cells were replated into an opaque 96-well tissue culture plate (Falcon, NJ) at a cell density of 40,000 cells/well per 100 µL media and assayed.

Plaque assays

10   To determine whether the adenoviral constructs described above replicate preferentially in melanocytes, plaque assays are performed. Plaquing efficiency is evaluated in the following cell types: melanoma cells (MeWo), prostate tumor cell lines (LNCaP), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3, SK-OV-3), and human embryonic kidney cells (293). 293 cells serve as a positive control for

15   plaquing efficiency, since this cell line expresses Ad5 E1A and E1B proteins. For analyzing constructs comprising a melanocyte-specific TRE, cells that allow a melanocyte-specific TRE to function, such as the cell lines provided above and cells that do not allow such function, such as HuH7, HeLa, PA-1, or G361, are used. The plaque assay is performed as follows: Confluent cell monolayers are seeded in 6-well dishes eighteen hours before

20   infection. The monolayers are infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the medium is removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques are scored two weeks after infection.

Example 13: Construction of a replication-competent adenovirus vector with a CEA-TRE and a EMCV IRES

Using a strategy similar to Example 1, the TRE fragment from Carcinembryonic antigen (CEA)(Table 3, SEQ ID NO:14) is used to construct virus designated CV873. A

5   PinAI fragment containing the CEA-TRE was cloned into the PinAI site in front of E1A CP627 for the transcriptional control. The resultant plasmid CP1080 is used together with pBHGE3 to generate CV873.

Example 14: *In vitro* and *In vivo* assays of anti-tumor activity

10   An especially useful objective in the development of urothelial cell-specific adenoviral vectors is to treat patients with bladder cancer. An initial indicator of the feasibility is to test the vector(s) for cytotoxic activity against cell lines and tumor xenografts grown subcutaneously in Balb/c nu/nu mice.

In vitro characterization of CV876

15   Virus yield assay for CV876

5 X 10^5 293, RT-4, SW780, PA-1, G361, MKNI, HBL-100, Fibroblast (from lung) and Smooth muscle cells (from bladder) were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at a MOI of 2 pfu/cell. After a

20   4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

25   Unlike wt. Ad5, CV802 which grows well in all of the cells tested, CV876 replicates much better in permissive cells (293, RT-4 and SW780) than in non-permissive cells (PA-1, G361, MKN1, HBL-100 and primary cells) by about 100-10000 fold. Noticeably, the replication in SW780 for CV876 is about 100 fold less than CV802, which indicates the limitation of this virus in efficacy.

30   Growth curve experiment for CV876

5 X 10^5 RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml

of serum-free RPMI 1640 containing CV802 (wt.Ad5 with 133) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw

5   cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV876 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. However, CV876 does show a delay of replication in RT-4 compared to CV802.

10   Cytopathic effect assay for CV876

5×10^5 293, RT-4, SW780, PA-1, MKN1 and LNCap were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium

15   was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV876 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (PA-1, MKN-1 and LNCap).

MTT assay for CV876

20   2×10^4 293, RT-4, SW780, MKN1, PA-1, HBL-100, Smooth muscle cells (from bladder) and Fibroblast (from lung) were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV876 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time point of day 1, 3,5,7 and 10. The medium was replaced

25   by 50ul of MTT at 1mg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV876 shows efficacy only in permissive cells

30   but not in non-permissive cells. Again, in RT-4 and SW780, CV876 kills the cells much slower than CV802.

In vitro characterization of CV882

<u>Virus yield assay for CV882</u>

5 X $10^5$293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later,

5   medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque

10   assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV882 in permissive cells (293, RT-4 and SW780) is comparable to CV802 (the difference is less than 100 fold) while it shows over 1000-1000000 fold difference in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells).

15   <u>Growth curve experiment for CV882</u>

5 X 10^5RT-4, PA-1, and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of

20   complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three, freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV882 replicates well only in RT-4 but not in

25   primary cells and PA-1 over a 120h period of time. Additionally, CV882 shows better replication in RT-4 compared to CV876.

<u>Cytopathic effect assay for CV882</u>

5 X $10^5$293, RT-4, SW780, HBL-100, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and

30   replaced with 1ml of serum-free RPNI 1640 containing CV802 (wt.Ad5 with E3) or CV882

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at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation
at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for
6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV882 only kills the permissive
5   cells (293, RT-4 and SW780) but not the non-permissive cells (HBL-100, G361, PA-1 and
Fibroblast cells).

MTT assay for CV882

2 X 10^{4}RT-4, SW780, PA-1, HBL-100, U118 and Fibroblast were plated into each
well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and
10 CV882 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric
assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10.
The medium was replaced by 50ul of MTT at 1mg/ml solution, which is converted to an
insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of
live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the
15 plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm
reference wavelength.

Similar as the results in CPE assay, CV882 shows efficacy only in permissive cells
but not in non-permissive cells.

In Vitro Characterization of CV884

20   Virus yield assay for CV884

5 X 10^{5}293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and
Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later,
medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802
(wt.Ad5 with E3) or CV984 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells
25 were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each
well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by
three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque
assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV884 is very similar as CV802 in permissive cells (293, RT-4
30 and SW780) but shows over 1000 fold difference with CV802 in non-permissive cells

(G361, LNCap, HBL-100, MKN1, PA-1 and primary cells). CV884 shows better efficacy than CV876 and CV882 without losing much specificity.

Growth curve experiment for CV884

5   5×10^5 RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw

10   cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV884 replicates very well only in RT-4 (similar as CV802) but not in primary cells and PA-1. Again, the replication of CV884 is better than CV882 and CV876.

15   Cytopathic effect assay for CV884

5×10^5 293, RT-4, SW780, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C,

20   medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV884 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (G361, PA-I and Fibroblast cells).

25   MTT assay for CV884

2×10^4 293, RT-4, SW780, U118, Fibroblast and Smooth muscle cells were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV884 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3,

30   5, 7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution which is

converted to an insoluble purple formazan by dehydrogenase enzymes present in active
mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by
isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength
and 690nm reference wavelength.

5   Similar as the results in CPE assay, CV884 shows strong efficacy (similar as
wt. Ad5) only in permissive cells but not in non-permissive cells.

In vivo activity of CV808

Mice were given subcutaneous (SC) injections of 1 x 10^6 sW780 cells. When
tumors grew to about 500 mm³, CV808 was introduced into the mice (5 X 10^7 PFU of virus
10   in 0.1 ml PBS and 10% glycerol) intratumorally. Control mice received vehicle alone.
Tumor sizes were measured weekly. The results are shown in FIG. 11. The data indicate
that CV808 was effective at suppressing tumor growth.

While it is highly possible that a therapeutic based on the viruses described here
would be given intralesionally (i.e., direct injection), it would also be desirable to
15   determine if intravenous (IV) administration of adenovirus vector can affect tumor growth.
If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits
inaccessible to direct injection. For this experiment, groups of mice bearing bladder
epithelial tumors are inoculated with 10^8 to 10^{10} PFU of an adenoviral vector by tail vein
injection, or with buffer used to carry the virus as a negative control. The effect of IV
20   injection of the adenoviral vector on tumor size is compared to vehicle treatment.

Example 15: Synergistic Effect of CV 890 with Chemotherapeutics
Materials and Methods

Cells. Hepatocellular carcinoma cell lines HepG2, Hep3B, PLC/PRF/5, SNU449,
25   and Sk-Hep-1, Chang liver cell (human normal liver cells), as well as other tumor cell lines
PA-1 (ovarian carcinoma), UM-UC-3 (bladder carcinoma), SW 780 (bladder carcinoma),
HBL100 (breast epithelia), Colo 201 (Colon adenocarcinoma), U 118 MG (glioblastoma)
and LNCaP (prostate carcinoma) were obtained from the American Type Culture
Collection. HuH-7 (liver carcinoma) was a generous gift of Dr. Patricia Marion (Stanford
30   University). 293 cells (human embryonic kidney containing the E1 region of Adenovirus)
were purchased from Microbix, Inc. (Toronto, Canada). The primary cells nBdSMC
(normal human bladder smooth muscle cells), nHLFC (normal human lung fibroblast

cells), and nHMEC (normal human mammary epithelial cells) were purchased from Clonetics (San Diego, California). All tumor cell lines were maintained in RPMI 1640 (BioWhittaker, Inc.) supplemented with 10% fetal bovine serum (Irvine Scientific), 100 U/ml penicillin and 100 ug/ml streptomycin. Primary cells were maintained in accordance

5   with vendor instructions (Clonetics, San Diego). Cells were tested for the expression of AFP by immunoassay (Genzyme Diagnostics, San Carlos, CA).

Virus yield and one-step growth curves. Six well dishes (Falcon) were seeded with 5×10^5 cells per well of calls of interest 24 hrs prior to infection. Cells were infected at an multiplicity of infection (MOI) of 2 PFU/cell for three hours in serum-free media. After 3

10   hours, the virus containing media was removed, monolayers were washed three times with PBS, and 4 ml of complete media (RPMI1640 + 10% FBS) was added to each well. 72 hours post infection, cells were scraped into the culture medium and lysed by three cycles of freeze-thaw.

The one-step growth curves time points were harvested at various time points after

15   infection. Two independent infections of each virus cell-combination were titered in duplicate on 293 cells (Yu et al., 1999, *Cancer Research*, 59:1498-1504.

Northern blot analysis. Hep3B or HBL100 cells were infected at an MOI of 20 PFU/cell (plaque forming unit per cell) with either CV802 or CV890 and harvested 24 hours post infection. Total cell RNA was purified using the RNeasy protocol (Qiagen).

20   The Northern blot was conducted using NorthernMax Plus reagents (Ambion, Austin, Texas). 5ug of RNA was fractionated on a 1% agarose, formaldehyde-based denaturing gel and transferred to a BrightStar-Plus (Ambion) positively charged membrane by capillary transfer. The antisense RNA probes for E1A (adenovirus genome 501bp to 1141bp) or E1B (1540bp-3910bp) were PCR products cloned in pGEM-T easy (Promega) and

25   transcription labeled with [α ^{32}P] UTP. Blots were hybridized at 68°C for 14 hours with ZipHyb solution and washed using standard methods (Ambion). Membranes were exposed to BioMax film (Kodak).

Western blot analysis. Hep3B or HBL100 cells were infected at MOI of 20 PFU/cell with either CV802 or CV890 and harvested 24 hours post infection. Cells were

30   washed with cold PBS and lysed for 30 min on ice in (50mM Tris, pH8.0, 150 mM NaCl, 1% IGEPAL CA360 a NP40 equivalent (Sigma), 0.5% sodium deoxycholate, and protease inhibitor cocktail from (Roche, Palo Alto, California). After 30 min centrifugation at 4C, the supernatant was harvested and the protein concentration determined with protein assay

ESL kit (Roche). Fifty micrograms of protein per lane were separated on 816% SDS-PAGE and electroblotted onto Hybond ECL membrane (Amersham Pharmacia, Piscataway, New Jersey). The membrane was blocked overnight in PBST (PBS with 0.1% Tween-20) supplemented with 5% nonfat dry milk. Primary antibody incubation was done

5   at room temperature for 2-3 hrs with PBST/1 % milk diluted antibody, followed by wash and 1 hr incubation with diluted horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California). Enhanced chemiluminescence (ECL; Amersham Pharmacia) was used for the detection. E1A antibody (clone M58) was from NeoMarkers (Fremont, California), E1B-21 kD antibody was from Oncogene

10   (Cambridge, Massachusetts). All antibodies were used according manufacturer's instruction.

Cell viability assay and statistical analysis. To determine the cell killing effect of virus and chemotherapeutic agent in combination treatment, a cell viability assay was conducted as previously described with modifications (Denizot, 1986, Journal

15   Immunology. Methods, 89:271-277). On 96 well plates, cells of interest were seeded at 10,000 calls per well 48 hr prior to infection. Cells were then treated with virus alone, drug alone, or in combination. Cell viability was measured at different time points by removing the media, adding 50 μl of 1mg/ml solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-^{2}H-tetrazolium bromide) (Sigma, St. Louis, MO) and incubating for 3 hrs at 37°C.

20   After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 50 μl of isopropanol followed by 30C incubation for 0.5 hr. The absorbency was determined on a microplate reader (Molecular Dynamics) at 560 nm (test wavelength) and 690 nm (reference wavelength). The percentage of surviving cells was estimated by dividing the OD_{550}-OD_{650} of virus or drug treated cells by the OD_{550}-OD_{650} of control cells.

one week prior to tumor implantation. Xenografts were established by injecting 1x10^6

Hep3B, HepG2 or LNCaP cells suspended in 100µl of RPMI 1640 media subcutaneously.

When tumors reached between 200 mm^3 and 300 mm^3, mice were randomized and dosed

with 100 µl of test article via intratumoral or the tail vein injection. Tumors were measured

5   in two dimensions by external caliper and volume was estimated by the formula [length

(mm) x width (mm)^2]/2. Animals were humanely killed when their tumor burden became

excessive. Serum was harvested weekly by retro-orbital bleed. The level of AFP in the

serum was determined by AFP Immunoassay kit (Genzyme Diagnostics, San Carlos, CA).

The difference in mean tumor volume and mean serum AFP concentration between

10   treatment groups was compared for statistical significance using the unpaired, two-tailed, *t*-

test.

Transcription and Translation of E1A/E1B Bicistronic Cassette of CV890 in Different Cells.

In wild type adenovirus infection, E1A and E1B genes produce a family of

15   alternatively spliced products. It has been found that there are five E1A mRNAs, among

them 12S (880 nucleotides, nts) and 13S (1018 nts) mRNAs are the dominant ones that are

expressed both early and late after infection. The 12S and 13S mRNAs encode the gene

product of 243 amino acids (243R) and 289 amino acids (289R) respectively (reviewed by

Shenk, 1996). The two major E1B transcripts that code for 19kD and 55kD proteins are

20   12S (1031 nts) and 22S (2287 nts) mRNAs. E1B 12S mRNA only codes the 19kD product,

whereas the 22S mRNA codes for both 19kD and 55kD products due to different initiation

sites during translation. In the current study, the generation of E1A-IRES-E1B bicistronic

cassette was expected to change the pattern of E1A and E1B transcripts in viral infection.

Therefore, Northern blot analysis was conducted to evaluate the steady-state level of E1A

25   and E1B transcripts. First, CV802 or CV890 were infected to Hep3B (AFP) or HBL100

(AFP) cells for 24 hours. The total RNA samples were separated on agarose gels and

processed for Northern blot by hybridizing to antisense RNA probes. The Northern blot

with E1A probe visualized the 12S and 13S mRNAs in both wild type CV802 infected

cells. For CV890, E1A transcripts can only be seen in Hep3B cells, indicating the

30   conditional transcription of E1A. It is of interest to find that in CV890, there is only one

large transcript (about 3.51Kb), whereas the 12S and 13S mRNAs are no longer present.

This large transcript indicates the continuous transcription of E1A-IRES-E1B bicistronic

cassette, suggesting an alteration of viral E1A splicing pattern in CV890. Transcription of

E1B from CV890 also appears to be AFP-dependent. It is clear that both 12S and 22S
mRNAs of E1B were present in wild type CV802 samples, whereas the 12S mRNA and an
enlarged 22S mRNA (3.5Kb) appeared in CV890 infected cells. Obviously, the identity of
this enlarged mRNA is the same 3.5Kb transcript as visualized in E1A blot, which is from

5   the transcription of E1A/E1B bicistronic cassette. Therefore, the E1B mRNA is tagged
after E1A mRNA in this large transcript. This large transcript contains all the coding
information for E1A, E1B 19kD and E1B 55kD. The mRNA splice pattern that appears in
CV802 is not valid in CV890, the 12S mRNA with E1B probe disappeared. Meanwhile, in
the E1B Northern blot, due to the selection of our E1B probe (1540bp-3910bp), mRNA of

10   the Adenovirus gene IX (3580bp-4070bp), the hexon-associated protein, was also detected.
In CV890 infected Hep3B cells, gene IX expression is equivalent to that of CV802,
whereas in CV890 infected HBL100, its expression was also completely shut down. This
result further demonstrated that the AFP controlled E1A/E1B expression is the key for late
gene expression as well as viral replication.

15   Results of the same samples in the Western blot also indicate that CV890 has AFP
dependent expression of E1A and E1B. Under our experimental conditions, E1A
expression level of CV890 in Hep3B cells is similar to that of CV802. However, when
E1B 19kD protein was detected, it was found that the expression level was much lower
than CV802 E1A. Previously, it has been addressed that IRES-mediated second gene has

20   less expression (Mizuguchi et al., 2000, *Mol. Ther.* 1:376-382). Taken together, CV890
infection in permissive Hep3B cells can produce normal amounts of E1A and lesser
amounts of E1B proteins capable of initiating a normal productive infection. In AFP⁻ cells,
however, this process was attenuated due to a lack of E1A and E1B gene transcription and
translation. These data demonstrated that the expression of both E1A and E1B genes are

25   under the control of AFP TRE and the artificial E1A/E1B bicistronic cassette is functioning
properly in CV890.

In Vitro Replication Specificity of CV890 in Tumor Cells and Primary Cells.

          From *in vitro* comparison of virus yield, CV890 has a better specificity profile than
CV732 (CV732 is an AFP-producing, cell-specific adenovirus variant in which the E1A

30   gene is under control of AFP-TRE). In order to gain further insights of using CV890 in
liver cancer therapy, more tumor cell lines and primary cells were tested to characterize *in
vitro* virus replication. First, all cells in the study were analyzed for their AFP status by
AFP immune assay. Based on AFP produced in the cells and media, all the cells were

divided into three groups, high (>2.5 µg/10^6 cells/10 days), low (<0.6 µg/10^6 cells/10 days) and none (undetectable in our study) (Table 7). It was confirmed that replication of CV890 in different cell lines correlates well with the AFP status of the host cell. Among the group of liver cell lines, CV890 only replicates well in AFP$^+$ cells, including Hep3B, HepG2,

5   Huh7, SNU449 and PLC/PRF/5. The amount of AFP required for the promoter activity seems very low as one of the hepatoma cell lines, SNU449, a previous reported AFP$^-$ cell (Park et al., 1995, *Int. J. Cancer* 62:276-282), produces very low AFP (about 60 ng/10^6 cells/10 days) compared to other cells. Nevertheless, even with very low amount of AFP, SNU449 cells can still support CV890 replication to the extent comparable to cells

10   producing significantly higher levels of AFP such as HepG2. Compared to CV802, CV890 is attenuated 5,000 to 100,000 fold in cells that do not produce AFP, including the hepatoma cell Sk-Hep1 and Chang liver cell, other tumor cells and primary cells. Taken together the results indicate that CV890 has shown a good specificity profile from a broad spectrum of tumor cells. Among them, only the AFP$^+$ liver cells, AFP production level

15   from high to low, are permissive for CV890.

          In another experiment, CV890 was compared to CV802 for their single step growth curves on different cells. Results demonstrated that CV890 has a similar growth kinetics to wild-type CV802 in AFP$^+$ cells except that virus yields are slightly lower (2-8 fold) in low AFP producing cells. In consideration of experimental error, there is no dramatic

20   difference in the replication of CV890 and CV802 in AFP$^+$ hepatoma cells. However, the growth curves of CV890 in AFP$^-$ cells showed clear attenuation. During a 5 day experiment, CV890 failed to replicate in AFP$^-$ cells including hepatoma cell (Change liver) and primary cells (nHLFC). From all the *in vitro* virus replication studies, it is clear that replication of CV890 is under the tight control of AFP-TRE and this adenovirus variant has

25   an excellent specificity profile of preferentially targeting AFP producing hepatocellular carcinoma cells.

          In Vivo Specificity and Efficacy of CV890.

          CV890 specificity was also evaluated in animals bearing prostate cancer LNCaP xenografts. In this *in vivo* test, nude mice with prostate xenograft were intravenously

30   injected with either CV890 or CV787, a prostate cancer specific adenovirus variant (Yu et al., 1999, Cancer Research, 59:4200-4203). Tumor volumes were documented and indicated that only CV787 had a significant antitumor efficacy in LNCaP xenografts, while tumors in the animals treated with CV890 grew, from 400 mm^3 to approximately 1200

mm^3 in six weeks, similar to the group treated with vehicle. This study indicates that CV890 does not attack LNCaP xenograft and keeps the good specificity profile under *in vivo* conditions.

To evaluate *in vivo* antitumor efficacy of CV890, different studies were carried out

5   in the nude mouse model harboring human hepatoma xenografts. First, BALB/c nu/nu mice with HepG2 or Hep3B xenografts were established, animals were further challenged with single dose or multiple doses of CV890 into the tumor mass (intratumoral administration, IT) or via their tail vein (intravenous administration, IV). Tumor volume and the level of serum AFP were monitored weekly after the start of treatment, and hence

10   the efficacy of the treatment was determined. The *in vitro* cytotoxicity study has demonstrated that CV890 has a better cytolytic effect than CV732. In order to further examine their antitumor activity, we first conducted animal study to compare CV890 to CV732. Animals harboring 300 mm^3 Hep3B xenograft were grouped (n=6) and injected with vehicle alone (control group), CV890 ($1x10^{11}$ particles/dose, CV890 group), or

15   CV732 ($1x10^{11}$ particles/dose, CV732 group). The Hep3B xenograft is a very aggressive tumor model and tumors grow very fast. Most animals can not survive long because of excessive tumor burden. During a six week study, single intravenous administration of CV890 have shown significant tumor growth inhibition, whereas control mice had over 10 fold tumor growth at week 5. In the group treated with CV732, single dose IV injection

20   also reduced the tumor growth as compared to control group, however, it was much less effective compared to CV890. For example, the average tumor volume of the CV890 treated group dropped from 312 mm^3 to 219 mm^3, while tumor volume increased from 308 mm^3 to 1542 mm^3 5 weeks after treatment in control. Both control group and the CV732 group were terminated at week 5 because excessive tumor size. Previously, CV732 has

25   been demonstrated to restrict the hepatoma tumor from growth after 5 doses of intravenous administration. Similar efficacy can be achieved with just a single intravenous administration of CV890, indicating that under *in vivo* conditions, CV890 has better efficacy than CV732 in hepatoma xenografts. In this experiment, 4 out of five CV890 treated mice were tumor free three weeks after treatment. However, in CV732 group,

30   xenografts in two mice stopped growing but none of treated animals were tumor free through the six-week experiment. There was no tumor reduction in this group or the control group of animals. By statistical analysis, the differences in mean relative tumor volumes and serum AFP concentrations between CV890 treated and CV732 treated or

vehicle treated tumors are significant (p<0.01)). Taken together, these studies suggest that CV890 has a significant antitumor activity and its oncolytic efficacy is better than CV732, an adenovirus variant similar to AvEla04I, in which the AFP TRE was applied to control E1A alone (Hallenback et al, 1999, Hum. Gene. Ther., 10:1721-1733).

5   Synergistic Antitumor Efficacy of CV890 in Combination with
           Chemotherapeutic Agents

           In this example, different chemotherapeutic agents were tested in combination with CV890 for their *in vitro* killing effect in Hep3B or HepG2 cells. Drug concentrations were optimized to the extent that they would not generate extensive cytotoxic effect on their

10   own. Under such conditions, some agents had shown higher cell killing effect in combination with CV890. Among them, doxorubicin, a drug currently used in treatment of HCC showed synergistic cytotoxicity with CV890. In experiments using doxorubicin together with CV890 on Hep3B cells, doxorubicin at 10ng/ml did not generate cytotoxicity, whereas CV890 at an MOI of 0.01 (pfu/cell) only had about 35% of cell killed at day 9.

15   However, when both were applied together, 90% cells were killed 9 days after treatment. In order to determine the potential synergistic effect from the combination treatment, the MTT cell viability data were subjected to further statistical analysis. Figure 10 shows a representative IC_{50} isobologram of doxorubicin and CV890 on Hep3B cells at day 5. First, the dose-response curves of doxorubicin alone or CV890 alone were made. Based on the

20   original theory of Steel and Peckham (1993) and method by Aoe et al. (1999), three isoeffect curves (mode I and mode 2a, 2b) were constructed. From this isobologram, several data points were in the synergy or additive area, indicating that combination of CV890 and doxorubicin provides synergistic effect on killing of Hep3B cells.

           Although CV890 alone has good antitumor activity, we applied combination

25   therapy with doxorubicin for *in vivo* evaluation of synergy. Animals harboring 300 mm^3 Hep3B xenografts were grouped (n=6) and injected with vehicle alone (control group), CV890 alone (1x10^{11} particles/dose, CV890 group), doxorubicin alone (10mg/kg, doxorubicin group), or CV890 in combination with doxorubicin (combination group). Figure 11 shows weekly change of the relative tumor size normalized to 100% at day 1. In

30   this experiment, by week six, all animals in the control group had excessive tumor which has increased by 700% of baseline, whereas in CV890 group and combination group, animals had either tumor free or tumor reduction. Of the eight Hep3B xenografts, treated with CV890, three animals (37.5%) had no palpable tumor at week 5, another three animals

had tumor regressed by more than 60%. In combination group, four out of eight animals were tumor free from week 5, another four animals had tumor reduction about 90%. All the animals in the CV890 and combination group were alive and tumor was suppressed even ten weeks following treatment whereas the control animals were sacrificed for

5   excessive tumor burden after week 6. Furthermore, CV890 also caused a drop in the serum AFP concentration in these mice. Statistical analysis shows that differences in mean relative tumor volumes and serum AFP concentrations between CV890 and vehicle treated group or combination and doxorubicin treated group are significant at different times (p<0.005).

10   The strong efficacy in the combination treatment shows that single IV injection of CV890 in combination of doxorubicin can eradicate aggressive Hep3B xenografts in most of the animals.

15   Table 7. AFP production in different tumor cells

                                       AFP

CELLS	(ng/10^6cells/10days)	
Hep3B	2645	
HepG2	3140	High
HuH7	4585	
SNU449	60	Low
PLC/PRF/5	600	
Chang	0	
SK-Hep1	0	
HBL100	0	None
PA-1	0	
LoVo	0	

TABLE 1 IRES SEQUENCES

> **SEQ ID NO:1** A 519 base pair IRES obtainable from encephelomycarditis virus (EMCV).

```
1     GACGTCGACTAATTCCGGTTATTTTCCACCATATTGCCGTCTTTTGGCAA
          SalI

51    TGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGG

101   GTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAG

151   GAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGAC

201   CCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCC

251   AAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGC

301   CACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAG

351   CGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGG

401   GATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGG

451   TTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGA
          SalI

501   AAAACACGATGTCGACGTC
```

> **SEQ ID NO:2** An IRES obtainable from vascular endothelial growth factor (VEGF).

```
1     ACGTAGTCGACAGCGCAGAGGCTTGGGGCAGCCGAGCGGCAGCCAGGCCC
           SalI

51    CGGCCCGGGCCTCGGTTCCAGAAGGGAGAGGAGCCCGCCAAGGCGCGCAA

101   GAGAGCGGGCTGCCTCGCAGTCCGAGCCGGAGAGGGAGCGCGAGCCGCGC

151   CGGCCCCGGACGGCCTCCGAAACCATGGTCGACACGTA
                                    SalI
```

> **SEQ ID NO:3** A 5'UTR region of HCV.

```
1     GCCAGCCCCCTGATGGGGGCGACACTCCGCCATGAATCACTCCCCTGTGAGGAACTACTG

61    TCTTCACGCAGAAAGCGTCTAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGAC

121   CCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAG

181   GACGACCGGGTCCTTTCTTGGATTAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCC

241   GCAAGACTGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG

301   GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACC (341)
```

> **SEQ ID NO:4** A 5'UTR region of BiP SEQ ID NO:4

```
1     CCCGGGGTCACTCCTGCTGGACCTACTCCGACCCCCTAGGCCGGGAGTGAAGGCGGGACT

61    TGTGCGGTTACCAGCGGAAATGCCTCGGGGTCAGAAGTCGCAGGAGAGATAGACAGCTGC

121   TGAACCAATGGGACCAGCGGATGGGGCGGATGTTATCTACCATTGGTGAACGTTAGAAAC
```

```
    181    GAATAGCAGCCAATGAATCAGCTGGGGGGGCGGAGCAGTGACGTTTATTGCGGAGGGGGC
    241    CGCTTCGAATCGGCGGCGGCCAGCTTGGTGGCCTGGGCCAATGAACGGCCTCCAACGAGC
    301    AGGGCCTTCACCAATCGGCGGCCTCCACGACGGGGCTGGGGGAGGGTATATAAGCCGAGT
    361    AGGCGACGGTGAGGTCGACGCCGGCCAAGACAGCACAGACAGATTGACCTATTGGGGTGT
5   421    TTCGCGAGTGTGAGAGGGAAGCGCCGCGGCCTGTATTTCTAGACCTGCCCTTCGCCTGGT
    481    TCGTGGCGCCTTGTGACCCCGGGCCCCTGCCGCCTGCAAGTCGAAATTGCGCTGTGCTCC
    541    TGTGCTACGGCCTGTGGCTGGACTGCCTGCTGCTGCCCAACTGGCTGGCAAGATG (595)
```

SEQ ID NO:5 A 5'UTR of PDGF SEQ ID NO:5

```
10  1      GTTTGCACCTCTCCCTGCCCGGGTGCTCGAGCTGCCGTTGCAAAGCCAACTTTGGAAAAA
    61     GTTTTTTGGGGGAGACTTGGGCCTTGAGGTGCCCAGCTCCGCGCTTTCCGATTTTGGGGG
    121    CTTTCCAGAAAATGTTGCAAAAAAGCTAAGCCGGCGGGCAGAGGAAAACGCCTGTAGCCG
    181    GCGAGTGAAGACGAACCATCGACTGCCGTGTTCCTTTTCCTCTTGGAGGTTGGAGTCCCC
    241    TGGGCGCCCCCACACCCCTAGACGCCTCGGCTGGTTCGCGACGCAGCCCCCCGGCCGTGG
15  301    ATGCTGCACTCGGGCTCGGGATCCGCCCAGGTAGCCGGCCTCGGACCCAGGTCCTGCGCC
    361    CAGGTCCTCCCCTGCCCCCCAGCGACGGAGCCGGGGCCGGGGGCGGCGGCGCCGGGGGCA
    421    TGCGGGTGAGCCGCGGCTGCAGAGGCCTGAGCGCCTGATCGCCGCGGACCTGAGCCGAGC
    481    CCACCCCCCTCCCCAGCCCCCCACCCTGGCCGCGGGGGCGGCGCGCTCGATCTACGCGTC
    541    CGGGGCCCCGCGGGGCCGGGCCCGGAGTCGGCATG (575)
```

20

TABLE 2 LITERATURE REFERENCES FOR IRES

IRES Host	Example	Reference
Picornavirus	HAV	Glass et al., 1993. Virol 193:842-852
	EMCV	Jang & Wimmer, 1990. Gene Dev 4:1560-1572
	Poliovirus	Borman et al., 1994. EMBO J 13:3149-3157
HCV and pestivirus	HCV	Tsukiyama-Kohara et al., 1992. J Virol 66:1476-1483
	BVDV	Frolov I et al., 1998. RNA. 4:1418-1435
Leishmania virus	LRV-1	Maga et al., 1995. Mol Cell Biol 15:4884-4889
Retroviruses	MoMLV VL30 (Harvey murine sarcoma virus)	Torrent et al., 1996. Hum Gene Ther 7:603-612
	REV	Lopez-Lastra et al., 1997. Hum Gene Ther 8:1855-1865
Eukaryotic mRNA	BiP	Macejak & Sarnow, 1991. Nature 353:90-94
	antennapedia mRNA	Oh et al., 1992. Gene & Dev 6:1643-1653
	FGF-2	Vagner et al., 1995. Mol Cell Biol 15:35-44
	PDGF-B	Bernstein et al., 1997. J Biol Chem 272:9356-9362
	IGFII	Teerink et al., 1995. Biochim Biophys Acta 1264:403-408
	eIF4G	Gan & Rhoads, 1996. J Biol Chem 271:623-626
	VEGF	Stein et al., 1998. Mol Cell Biol 18:3112-3119; Huez et al., 1998. Mol Cell Biol 18:6178-6190

5

TABLE 3 TRE SEQUENCES

Nucleotide sequence of a human uroplakin II 5' flanking region. Position +1 (the translational start site) is denoted with an asterisk. SEQ ID NO:6 (number 1 of SEQ ID NO:6 corresponds to position -2239 with respect to the translational start site).

	TCGATAGGTA	CCCACTATAG	GGCACGCGTG	GTCGACGGCC	CGGGCTGGTC
	1				50
5	TGGCAACTTC	AAGTGTGGGC	CTTTCAGACC	GGCATCATCA	GTGTTACGGG
	51				100
	GAAGTCACTA	GGAATGCAGA	ATTGATTGAG	CACGGTGGCT	CACACCTGTA
10	101				150
	ATCCCAACAC	TCTGGGAGGC	CAAGGCAGGT	GGATCACTTG	TGGTCAGGAG
	151				200
	TTTGAGACCA	GCCTGGCCAA	CATGGTGAAA	CCTCATCTCT	ACTAAAAATA
15	201				250
	CAAAAATTAG	CTGGGAATGG	TGGCACATGC	CTATAATCCC	AGTTACTCAG
	251				300
20	GAGGCTGAGG	CAGGAGAATC	ATTTGAACCT	GGGAGGCAGA	GGTTGCAGTG
	301				350
	AGCCGAGATC	ACGCCACTGC	ACTCCAGCCT	GGGTGACACA	GCGAGACTCT
	351				400
25	GTCTCAAAAA	AAAAAAAATG	CAGAATTTCA	GGCTTCACCC	CAGACCCACT
	401				450
	GCATGACTGC	ATGAGAAGCT	GCATCTTAAC	AAGATCCCTG	GTAATTCATA
30	451				500
	CGCATATTAA	ATTTGGAGAT	GCACTGGCGT	AAGACCCTCC	TACTCTCTGC
	501				550
35	TTAGGCCCAT	GAGTTCTTCC	TTTACTGTCA	TTCTCCACTC	ACCCCAAACT
	551				600
	TTGAGCCTAC	CCTTCCCACC	TTGGCGGTAA	GGACACAACC	TCCCTCACAT
	601				650
40	TCCTACCAGG	ACCCTAAGCT	TCCCTGGGAC	TGAGGAAGAT	AGAATAGTTC
	651				700
	GTGGAGCAAA	CAGATATACA	GCAACAGTCT	CTGTACAGCT	CTCAGGCTTC
	701				750
45	TGGAAGTTCT	ACAGCCTCTC	CCGACAAAGT	ATTCCACTTT	CCACAAGTAA
	751				800
	CTCTATGTGT	CTGAGTCTCA	GTTTCCACTT	TTCTCTCTCT	CTCTCTCTCT
50	801				850

```
     CAACTTTCTG   AGACAGAGTT   TCACTTAGTC   GCCCAGGCTG   GAGTGCAGGG
     851                                                        900

     GCACAATCTC   GGCTCACTGC   AACCTCCACC   TCCTGGGTTC   AAGTGTTTCT
5    901                                                        950

     CCTGTCTCAG   CCTCCCGAGT   AGCTGGGATT   ACAGGCACAC   ACCACCGCGT
     951                                                        1000

10   TAGTTTTTGT   ATTTTTGGTA   GAGATGGTGT   TTCGCCATAT   TGGCCAGGCT
     1001                                                       1050

     GATCTCGAAC   TCCTGACCTC   AGGTGATCCG   CCCACCTCGG   CCTCCCAAAG
     1051                                                       1100
15
     TGCTGGGATT   ACAGGCATGA   GCCACCACGC   CCGGCTGATC   TCTTTTCTAT
     1101                                                       1150

     TTTAATAGAG   ATCAAACTCT   CTGTGTTGCC   TAGGCTGGTC   TTGAACTCCT
20   1151                                                       1200

     GGCCTCGAGT   GATCCTCCCA   CCTTGGCCTC   CCAAAGTGTT   GAGATTACAG
     1201                                                       1250

25   GCATGAGCCA   CTGTGCCTGG   CCTCAGTTCT   ACTACAAAAG   GAAGCCAGTA
     1251                                                       1300

     CCAGCTACCA   CCCAGGGTGG   CTGTAGGGCT   ACAATGGAGC   ACACAGAACC
     1301                                                       1350
30
     CCTACCCAGG   GCCCGGAAGA   AGCCCCGACT   CCTCTCCCCT   CCCTCTGCCC
     1351                                                       1400

     AGAACTCCTC   CGCTTCTTTC   TGATGTAGCC   CAGGGCCGGA   GGAGGCAGTC
35   1401                                                       1450

     AGGGAAGTTC   TGTCTCTTTT   TCATGTTATC   TTACGAGGTC   TCTTTTCTCC
     1451                                                       1500

40   ATTCTCAGTC   CAACAAATGG   TTGCTGCCCA   AGGCTGACTG   TGCCCACCCC
     1501                                                       1550

     CAACCCCTGC   TGGCCAGGGT   CAATGTCTGT   CTCTCTGGTC   TCTCCAGAAG
     1551                                                       1600
45
     TCTTCCATGG   CCACCTTCGT   CCCCACCCTC   CAGAGGAATC   TGAAACCGCA
     1601                                                       1650

     TGTGCTCCCT   GGCCCCCACA   GCCCCTGCCT   CTCCCAGAGC   AGCAGTACCT
50   1651                                                       1700
```

```
     AAGCCTCAGT  GCACTCCAAG  AATTGAAACC  CTCAGTCTGC  TGCCCCTCCC
     1701                                                      1750

     CACCAGAATG  TTTCTCTCCC  ATTCTTACCC  ACTCAAGGCC  CTTTCAGTAG
  5  1751                                                      1800

     CCCCTTGGAG  TATTCTCTTC  CTACATATCA  GGGCAACTTC  CAAACTCATC
     1801                                                      1850

 10  ACCCTTCTGA  GGGGTGGGGG  AAAGACCCCC  ACCACATCGG  GGGAGCAGTC
     1851                                                      1900

     CTCCAAGGAC  TGGCCAGTCT  CCAGATGCCC  GTGCACACAG  GAACACTGCC
     1901                                                      1950
 15
     TTATGCACGG  GAGTCCCAGA  AGAAGGGGTG  ATTTCTTTCC  CCACCTTAGT
     1951                                                      2000

     TACACCATCA  AGACCCAGCC  AGGGCATCCC  CCCTCCTGGC  CTGAGGGCCA
 20  2001                                                      2050

     GCTCCCCATC  CTGAAAAACC  TGTCTGCTCT  CCCCACCCCT  TTGAGGCTAT
     2051                                                      2100

 25  AGGGCCCAAG  GGGCAGGTTG  GACTGGATTC  CCCTCCAGCC  CCTCCCGCCC
     2101                                                      2150

     CCAGGACAAA  ATCAGCCACC  CCAGGGGCAG  GGCCTCACTT  GCCTCAGGAA
     2151                                                      2200
 30
     CCCCAGCCTG  CCAGCACCTA  TTCCACCTCC  CAGCCCAGCA
     2201                               2239
```

Nucleotide sequence of a mouse uroplakin II 5' flanking region. The translational

35 start site is denoted with an asterisk. SEQ ID NO:7 (number 1 of SEQ ID NO:7

corresponds to position -3592 with respect to the translational start site).

```
     CTCGAGGATCTCGGCCCTCTTTCTGCATCCTTGTCCTAAATCATTTTCAT
 40  1                                                   50

     ATCTTGCTAGACCTCAGTTTGAGAGAAACGAACCTTCTCATTTTCAAGTT
     51                                                  100

 45  GAAAAAAAAAGAGGTTCAAAGTGGCTCACTCAAAGTTACAAGCCAACAC
     101                                                 150

     TCACCACTACGAGTACAATGGCCACCATTAGTGCTGGCATGCCCCAGGAG
     151                                                 200
 50
```

ACAGGCATGCATATTATTCTAGATGACTGGGAGGCAGAGGGGTGGCCTAG
201   250

TGAGGTCAGACTGTGGACAGATCAGGCAGATGTGGGTTCTGATCCCAATT
251   300

CCTCAGGCCGCAGAACTACTGTGGTTCAAGAAGGGGACAAAAGGACTGCA
301   350

GTCCGGAACAGGAGGTCCATTTGAGAGCTGACTGAGCAGAAGAGGAAAGT
351   400

GAAGAACTTCTGGGGCAAGAGCTTACCCTACTTTACAGCTTTGTTGTCTT
401   450

CTTTACTCCAGGGGCGTCCCTGGTACTCAGTAAATGTCTGTTGGCTTGAG
451   500

GAACATATGTGTAAGGAGGAAGGAGAGGGAACTTGAGGGAGTTAAGACTC
501   550

AAGAATCAATCAAGGAGAGGACAGCAGAGAAGACAGGGTTTGGGAGAGAG
551   600

ACTCCAGACATTGGCCCTGGTTCCCTTCTTGGCCACTGTGAAACCCTCCA
601   650

GAGGAACTGAGTGCTGTGGCTTTAAATGATCTCAGCACTGTCAGTGAAGC
651   700

GCTCTGCTCAAAGAGTTATCCTCTTGCTCCTGTGCCGGGGCCTCCCCCTC
701   750

CTCTCAGCTCCCAAACCCTTCTCAGCCACTGTGATGGCATAATTAGATGC
751   800

GAGAGCTCAGACCGTCAGGTCTGCTCCAGGAACCACCCATTTTCCCCAAC
801   850

CCCAGAGAAAGGTCCTAGTGGAAAAGTGGGGGCCACTGAAGGGCTGATGG
851   900

GGTTCTGTCCTTTCCCCCATGCTGGGTGGACTTAAAGTCTGCGATGTGTG
900   950

TAGGGGGTAGAAGACAACAGAACCTGGGGGCTCCGGCTGGGAGCAGGAGG
951   1000

AACTCTCACCAGACGATCTCCAAATTTACTGTGCAATGGACGATCAGGAA
1001   1050

ACTGGTTCAGATGTAGCTTCTGATACAGTGGGTCTGAGGTAAAACCCGAA
1051   1100

ACTTAATTTCTTTCAAAAATTTAAAGTTGCATTTATTATTTTATATGTGT
1101   1150

GCCCATATGTGTGCCACAGTGTCTATGTGGAGGTCAGAGGGCAAGTTGTG
1151   1200

5 GGCATTGGCTCTCTCCTTTCATAATGTGGCTTCTGGGGACCAAAATGTCA
1201   1250

GGCATGGTGGCAAGAGCTTTTACCTGTTGAGCCATCTCATGGTTTCGTAA
1251   1300
10 AACTTCCTATGACGCTTACAGGTAACGCAGAGACACAGACTCACATTTGG
1301   1350

AGTTAGCAGATGCTGTATTGGTGTAAACACTCATACACAGACACACACAC
15 1351`   1400

ATACTCATACACACACACACACTTATCACATGCACACACATACTCGTA
1401   1450

20 TACACACAGACACACACACATGCACTCTCACATTCACATATTCATACACA
1451   1500

TCCACACACACACTCATCCACACACACAGACACACATACTCATCCACACA
1501   1550
25
CACACACACACATACTCATACACACACACAGACACACATACTCATACACA
1551   1600

CACACAGACACACACATATAATCATACATACACAGACACACTCATACATG
30 1601   1650

TGCACACACACTCATCCACACACACACACTCATACACACACACTCA
1651   1700

35 TACACACACACTCATACACACACACACGAGGTTTTTCTCAGGCTGCCT
1701   1750

TTGGGTGGAGACTGGAACTGATTTCTGTTTTTCAGCTCCTTGGCTTTTTG
1751   1800
40
TCCCTTTAGATGAGATCTCCTCCTCACTTTACACACAGAAAGATCACACA
1801   1850

CGAGGGAGAACTGGCGGTGCGGAAGAGGGCTACACGGTAGGGTGTCAGGG
45 1851   1900

TCAGGAGATCTTCCTGGCAAGTCTCAAACCTCCACATAGCACAGTGTTTA
1901   1950

50 CGTGAGGATTTAGGAGGAATCAGGAAGAGGATTGGTTTACTGCAGAGCAG
1951   2000

ACCATATAGGTCCACTCCTAAGCCCCATTTGAAATTAGAAGTGAGACAGT
2001   2050
55
GTGGGATAAAAAGAGCAGATCTCTGGTCACATTTTTAAAGGGATATGAGG

2051   3000

GTCCTGTGCCTTTAAGCCTTCCCATCTCCCTCCAATCCCCCCTCACCTTC
2101   2150

CCCACCCTAACCCTCCCCAGGTTTCTGGAGGAGCAGAGTTGCGTCTTCTC
2151   2200

CCTGCCCTGCCGAGCTGCTCACTGGCTGCTCTAGAGGCTGTGCTTTGCGG
2201   2250

TCTCCATGGAAACCATTAGTTGCTAAGCAACTGGAGCATCATCTGTGCTG
2251   2300

AGCTCAGGTCCTATCGAGTTCACCTAGCTGAGACACCCACGCCCCTGCAG
2301   2350

CCACTTTGCAGTGACAAGCCTGAGTCTCAGGTTCTGCATCTATAAAAACG
2351   2400

AGTAGCCTTTCAGGAGGGCATGCAGAGCCCCCTGGCCAGCGTCTAGAGGA
2401   2450

GAGGTGACTGAGTGGGGCCATGTCACTCGTCCATGGCTGGAGAACCTCCA
2451   2500

TCAGTCTCCCAGTTAGCCTGGGGCAGGAGAGAACCAGAGGAGCTGTGGCT
2501   2550

GCTGATTGGATGATTTACGTACCCAATCTGTTGTCCCAGGCATCGAACCC
2551   2600

CAGAGCGACCTGCACACATGCCACCGCTGCCCCGCCCTCCACCTCCTCTG
2601   2650

CTCCTGGTTACAGGATTGTTTTGTCTTGAAGGGTTTTGTTGTTGCTACTT
2651   2700

TTTGCTTTGTTTTTTCTTTTTTAACATAAGGTTTCTCTGTGTAGCCCTAG
2701   2750

CTGTCCTGGAACTCACTCTGTAGACCAGGCTGGCCTCAAACTCAGAAATC
2751   2800

CACCTTCCTCCCAAGTGCTGGGATTAAAGGCATTCGCACCATCGCCCAGC
2801   2850

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2951   3000

102

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AFP-TRE. SEQ ID NO:8.

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121  AAAATTTGCTGTTCTTCATGGTTTCTCTTTTCACTGCTATCTATTTTTCTCAACCACTCA
181  CATGGCTACAATAACTGTCTGCAAGCTTATGATTCCCAAATATCTATCTCTAGCCTCAAT

241  CTTGTTCCAGAAGATAAAAAGTAGTATTCAAATGCACATCAACGTCTCCACTTGGAGGGC

301  TTAAAGACGTTTCAACATACAAACCGGGGAGTTTTGCCTGGAATGTTTCCTAAAATGTGT

361  CCTGTAGCACATAGGGTCCTCTTGTTCCTTAAAATCTAATTACTTTTAGCCCAGTGCTCA

421  TCCCACCTATGGGGAGATGAGAGTGAAAAGGGAGCCTGATTAATAATTACACTAAGTCAA
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481   TAGGCATAGAGCCAGGACTGTTTGGGTAAACTGGTCACTTTATCTTAAACTAAATATATC

541   CAAAACTGAACATGTACTTAGTTACTAAGTCTTTGACTTTATCTCATTCATACCACTCAG

601   CTTTATCCAGGCCACTTATGAGCTCTGTGTCCTTGAACATAAAATACAAATAACCGCTAT

661   GCTGTTAATTATTGGCAAATGTCCCATTTTCAACCTAAGGAAATACCATAAAGTAACAGA

721   TATACCAACAAAAGGTTACTAGTTAACAGGCATTGCCTGAAAAGAGTATAAAAGAATTTC

781   AGCATGATTTTCCATATTGTGCTTCCACCACTGCCAATAACA (822)

Probasin-TRE SEQ ID NO:9

-426
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                               ARE-1
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ATTGGGCATTGTCCATGCC<u>TAGTAAAGTACTCCAAGAACCTATTTGTATACTA</u>
                   ARE-2
GATGACACAATGTCAATGTCTGTGTACAACTGCCAACTGGGATGCAAGACAC

TGCCCATG<u>**CCAAT**</u>CATCCTGAAAAGCAGC<u>**TATAAAAAG**</u>CAGGAAGCTACTCT
        CAAT box   TATAA box

+1   +28
GCAC<u>C</u>TTGTCAGTAGGTCCAGATACCTACAG-3'
Transcription site

Tyrosinase-TRE. SEQ ID NO:10
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1   <u>CCGGT</u>(T)GAAAATGATAAGTTGAATTCTGTCTTCGAGAACATAGAAAAGAA

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101 AGGGACAAGAAGTCTTGTGTTAACTCTTTGTGGCTCTGAAAGAAAGAGAG

151 AGAGAAAAGATTAAGCCTCCTTGTGGAGATCATGTGATGACTTCCTGATT
                                              -1716
201 -231(C)CAGCCAGAGCGAGCATTTCCATGGAAACTTCTCTTCCTCTTC(A)CTCGAG

251 (A)TTACTAACCTTATTGTTAATATTCTAACCATAAGAATTAAACTATTAAT

301 GGTGAATAGAGTTTTTCACTTTAACATAGGCCTATCCCACTGGTGGGATA

351 CGAGCCAATTCGAAAGAAAAAGTCAGTCATGTGCTTTTCAGAGGATGAA(A) +65

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401   GCTTAAGATAAAGACTAAAAGTGTTTGATGCTGGAGGTGGGAGTGGTATT
451   ATATAGGTCTCAGCCAAGACATGTGATAATCACTGTAGTAGTAGCTGGAA
501   AGAGAAATCTGTGACTCCAATTAGCCAGTTCCTGCAGACCTTGTGA
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PinAl end

Human glandular kallikrein-TRE SEQ ID NO:11

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127

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aaca                                                                   5224
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CLAIMS

What is claimed is:

1.   A replication-competent adenovirus vector comprising first and second
genes co-transcribed as a single mRNA wherein the first and the second genes are under
transcriptional control of a heterologous, target cell-specific transcriptional regulatory
element (TRE), wherein the second gene has a mutation in or deletion of its endogenous
promoter and is under translational control of an internal ribosome entry site (IRES) and
wherein said vector exhibits greater specificity for the target cell than an adenovirus vector
comprising a target cell-specific TRE operably linked to a gene and lacking an IRES.

2.   The vector of Claim 1, wherein at least one of said first and second genes is
an adenovirus gene.

3.   The vector of Claim 2, wherein both of said first and said second genes are
adenovirus genes.

4.   The vector of Claim 2, wherein at least one of said first and said second
adenovirus gene is essential for viral replication.

5.   The vector of Claim 4, wherein the adenovirus gene essential for viral-
replication is an adenovirus early gene.

6.   The vector of Claim 5, wherein the adenovirus early gene includes E1A,
E1B, E2, or E4.

7.   The vector of Claim 4, wherein the adenovirus gene essential for viral
replication is an adenovirus late gene.

8.   The vector of Claim 3, wherein both said first and said second adenovirus
genes are essential for viral replication.

9.   The vector of Claim 8, wherein at least one of said first and said second adenovirus genes is an adenovirus early gene.

10.   The vector of Claim 8, wherein at least one of said first and said second adenovirus genes is an adenovirus late gene.

11.   The vector of Claim 8, wherein said first adenovirus gene is E1A and said second adenovirus gene is E1B.

12.   The vector of Claim 11 wherein E1A has its endogenous promoter deleted.

13.   The vector of Claim 11 wherein E1A has an inactivation of E1A enhancer I

14.   The vector of Claim 11 wherein E1B has an inactivation of its endogenous promoter.

15.   The vector of Claim 11 wherein E1B has a deletion of the 19-kDa region.

16.   The vector of Claim 11 wherein E1A has an inactivation of its endogenous promoter and E1B has an inactivation of its endogenous promoter.

17.   The vector of Claim 16 wherein E1B has a deletion of the 19-kDa region.

18.   The vector of Claim 16 wherein E1A has an inactivation of E1A enhancer I.

19.   The vector of Claim 1, wherein the internal ribosome entry site (IRES) is from EMCV.

20.   The vector of Claim 1 wherein the IRES is from VEGF.

21.   The vector of Claim 1 wherein the IRES includes the 5'UTR of HCV; the 5' UTR of BiP; or the 5'UTR of PDGF.

130

22.   The vector of Claim 1, wherein the TRE is specific for a target cell that is a cancer cell.

5   23.   The vector of Claim 22 wherein the cancer cell includes a prostate cancer cell, a breast cancer cell, a hepatoma cell, a melanoma cell, a bladder cell or a colon cancer cell.

24.   The vector of Claim 22, wherein the TRE includes the probasin (PB) TRE,

10   the prostate-specific antigen (PSA) TRE, the mucin (*MUC1*) TRE, the α-fetoprotein (AFP) TRE, the *hKLK2* TRE, the tyrosinase TRE, the human uroplakin II (hUPII) TRE or the carcinoembryonic antigen (CEA) TRE.

25.   The vector of Claim 9 wherein said first adenovirus gene has a deletion of

15   its endogenous promoter.

26.   The vector of Claim 25 wherein said first adenovirus gene is E1A.

27.   The vector of Claim 9 wherein said first and/or said second adenovirus gene

20   has a deletion of an enhancer region.

28.   The vector of Claim 27 wherein said first gene is E1A and said enhancer is E1A enhancer I.

25   29.   The vector of Claim 1 wherein said TRE has an endogenous silencer element deleted.

30.   The vector of Claim 1 wherein said adenovirus vector comprises an E3 region.

30

31.   The adenovirus vector of Claim 11 wherein said adenovirus comprises an E3 region.

32.   The adenovirus vector of Claim 11 further comprising a transgene.

33.   An adenovirus vector comprising a gene under transcriptional control of a melanocyte-specific TRE.

5

34.   The vector of Claim 33 wherein said gene is an adenoviral gene.

35.   The vector of Claim 34 wherein said adenoviral gene is a gene essential for replication.

10

36.   The vector of Claim 32 wherein said transgene is co-transcribed with said first and said second gene and said transgene is under the translation control of a separate internal ribosome entry site (IRES).

15   37.   The vector of Claim 36 wherein said IRES is from EMCV.

38.   The vector of Claim 36 wherein said IRES is from VEGF.

39.   The vector of Claim 30 further comprising an adenovirus death protein gene
20   (ADP).

40.   The vector of Claim 32 wherein said transgene is a cytotoxic gene.

41.   The vector of Claim 1 wherein said first adenovirus gene is essential for
25   viral replication and said second adenovirus gene is the adenovirus death protein gene (ADP).

42.   The vector of Claim 41 wherein said first adenovirus gene is E1A.

30   43.   The vector of Claim 42 wherein E1A has a deletion of its endogenous promoter.

44.   The vector of Claim 42 wherein said E1A has a deletion of E1A enhancer I.

45.   The vector of Claim 1 wherein said first gene is essential for viral replication and said second gene is E3.

5   46.   The vector of Claim 45 wherein said first gene is E1A.

47.   A composition comprising a vector according to Claim 1.

48.   The composition of Claim 47 further comprising a pharmaceutically
10   acceptable excipient.

·49.   A composition comprising a vector according to Claim 30.

50.   The composition of Claim 49 further comprising a pharmaceutically
15   acceptable excipient.

51.   A host cell comprising the vector of Claim 1.

52.   A host cell comprising the vector of Claim 30.
20

53.   An adenovirus vector comprising E1B under transcriptional control of a heterologous, target cell specific TRE, wherein E1B has a deletion of part or all of the 19-kDa region.

25   54.   A host cell comprising the adenovirus vector of claim 53.

55.   A method for propagating a replication-competent adenovirus vector comprising a target cell-specific TRE, said method comprising combining an adenovirus vector of Claim 1 with mammalian cells that permit the function of a target cell-specific
30   TRE, such that the adenovirus vector enters the cell, whereby said adenovirus vector is propagated.

56.   A method for conferring selective cytotoxicity in target cells, comprising contacting the cells with an adenovirus vector of Claim 41 whereby the vector enters the cell.

5   57.   A method for modifying the genotype of a target cell, comprising contacting the cell with an adenovirus vector of Claim 1, wherein the vector enters the cell.

58.   A method for suppressing tumor cell growth, comprising contacting a tumor cell with an adenovirus vector of Claim 41 such that the adenovirus vector enters the tumor
10   cell and exhibits selective cytotoxicity for the tumor cell.
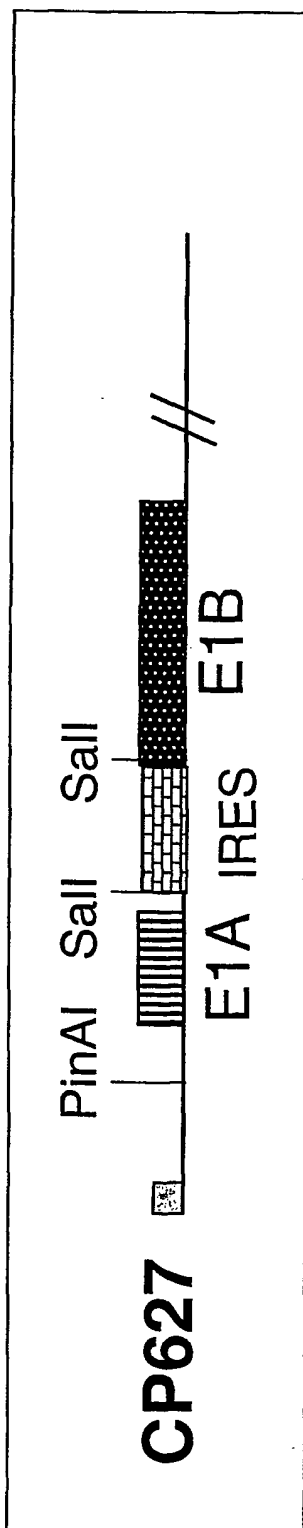
Figure 1

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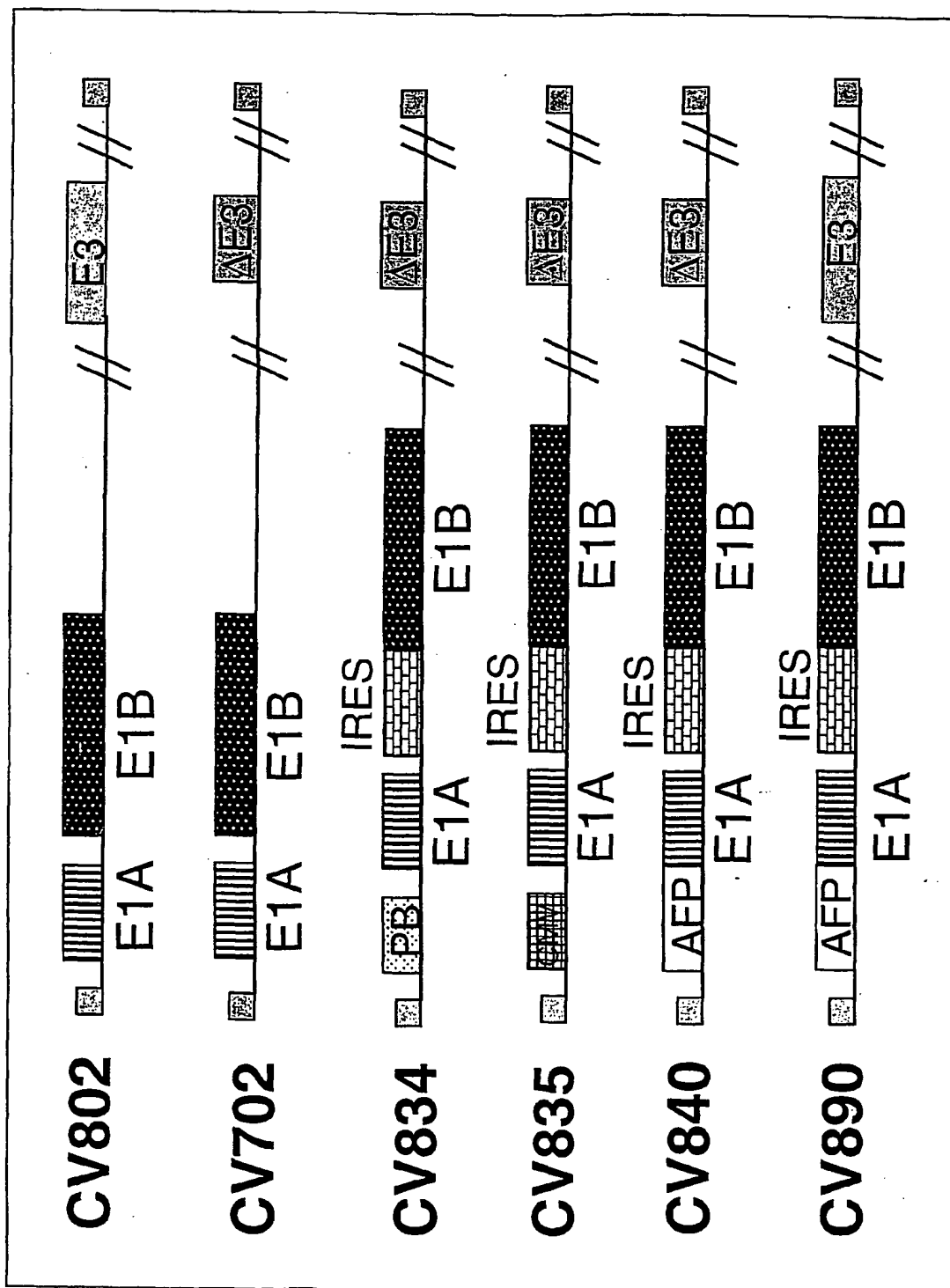
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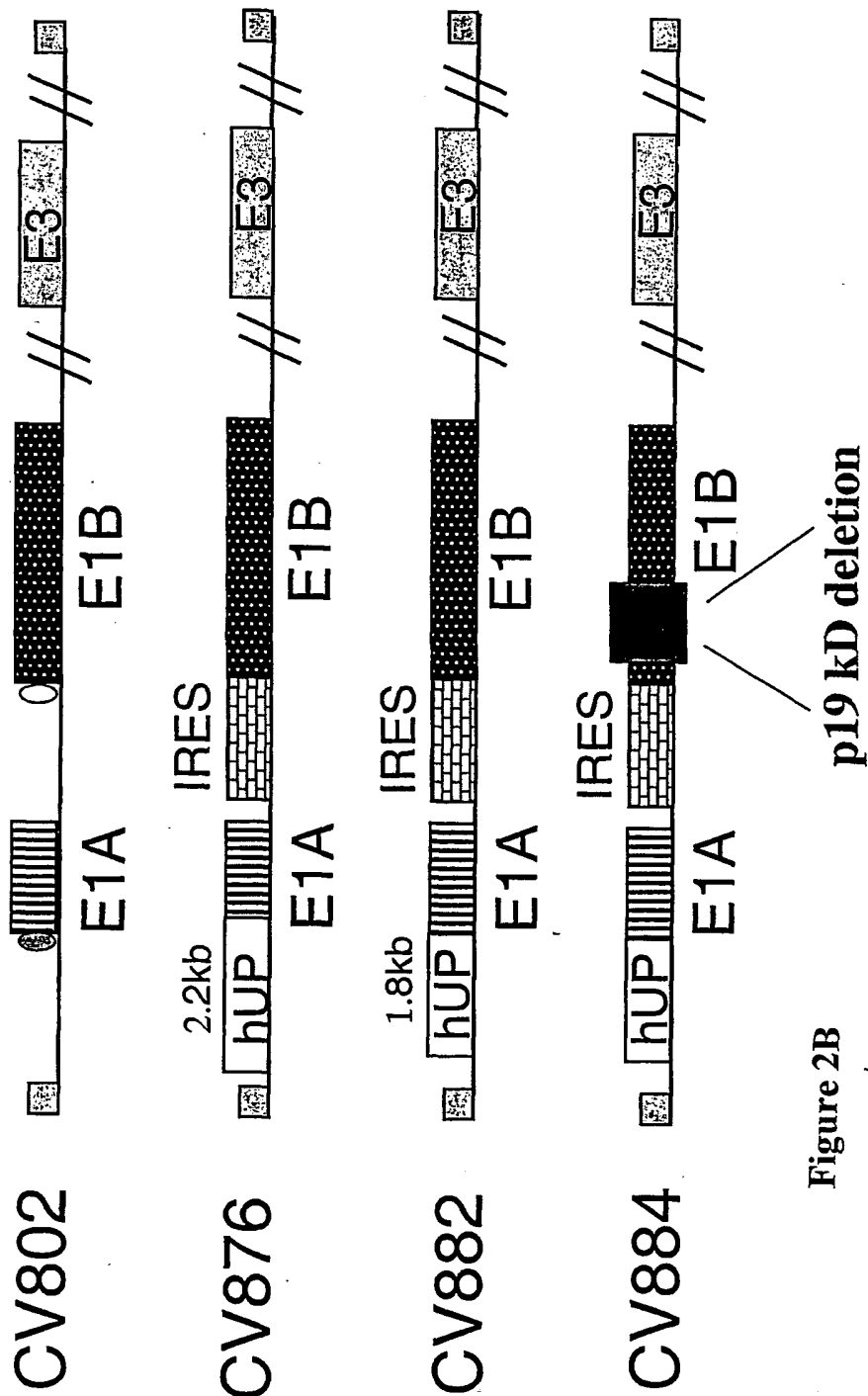
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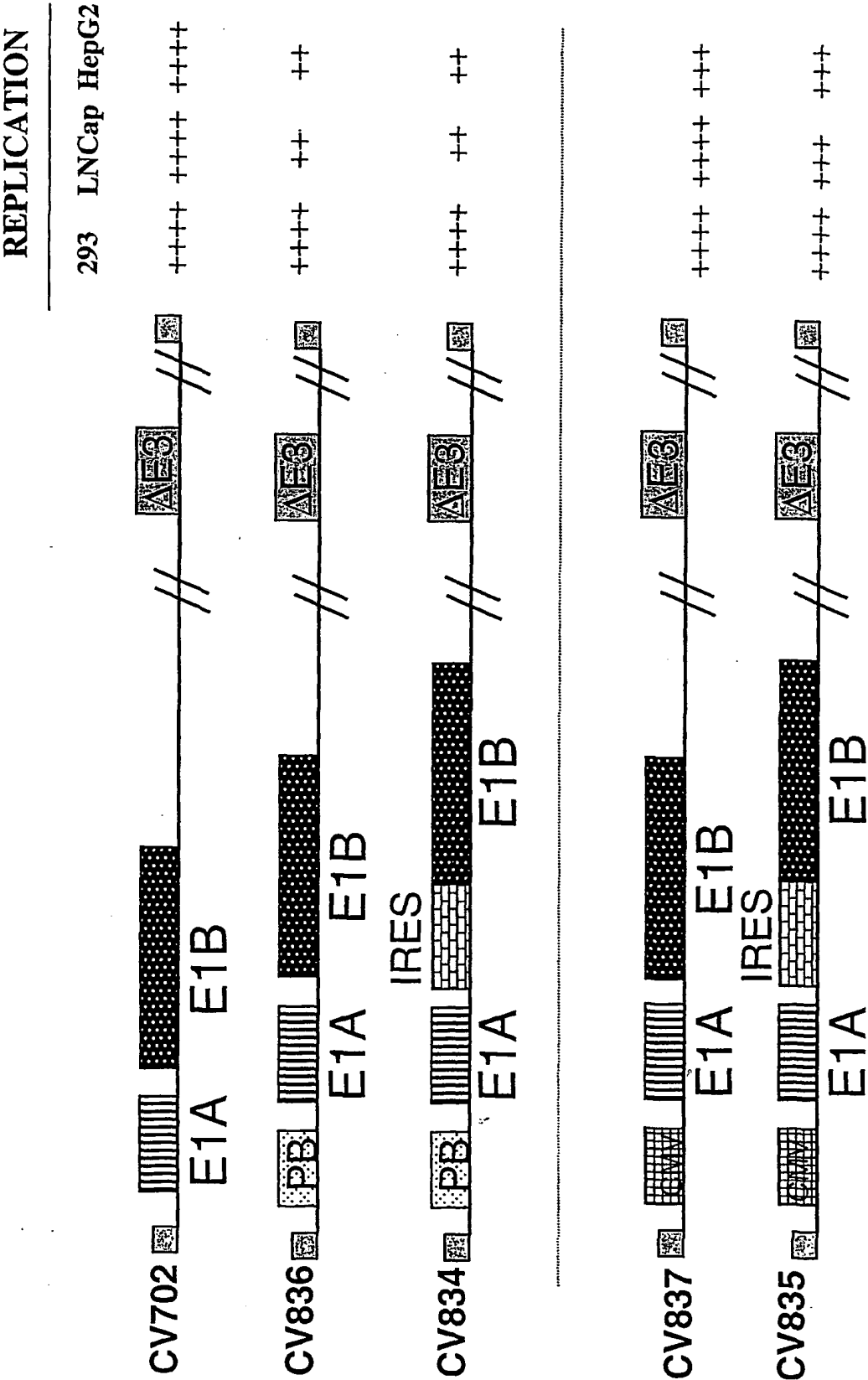
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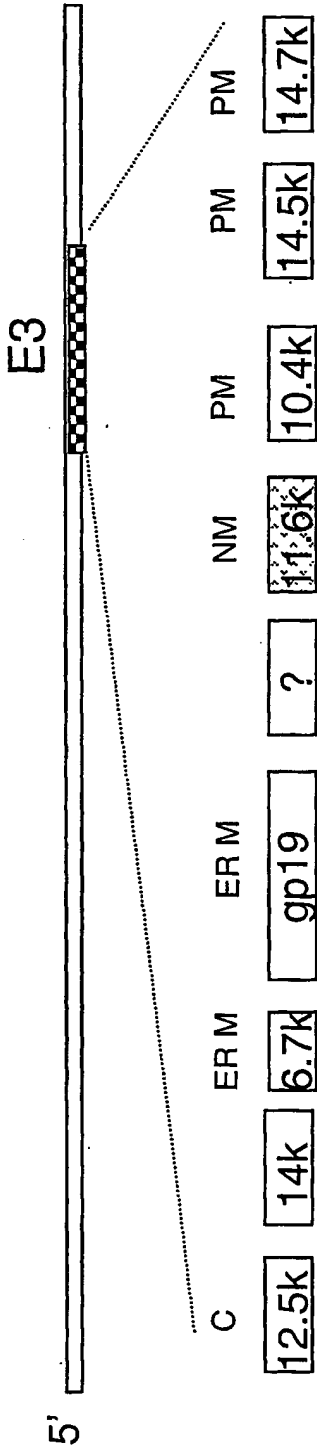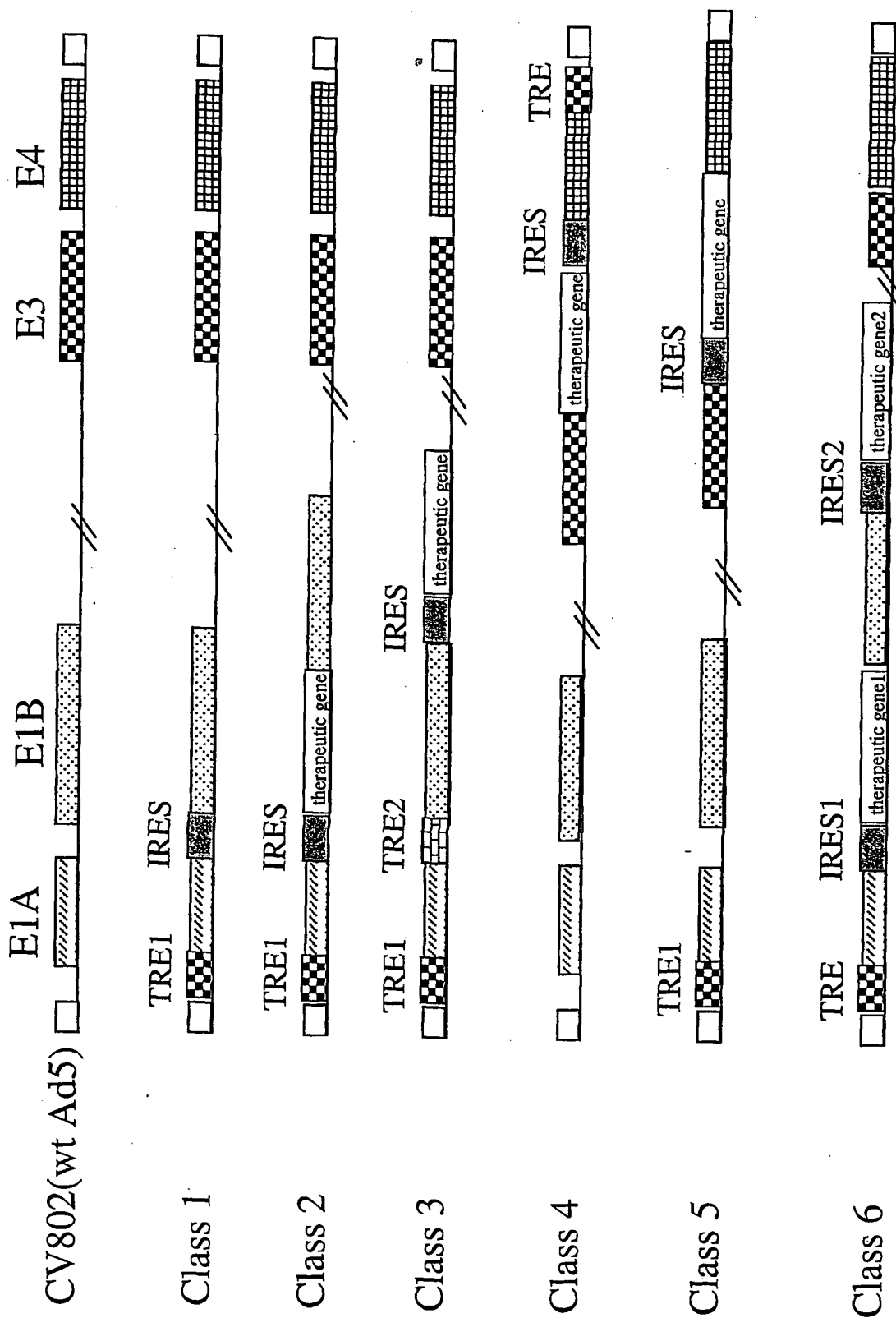
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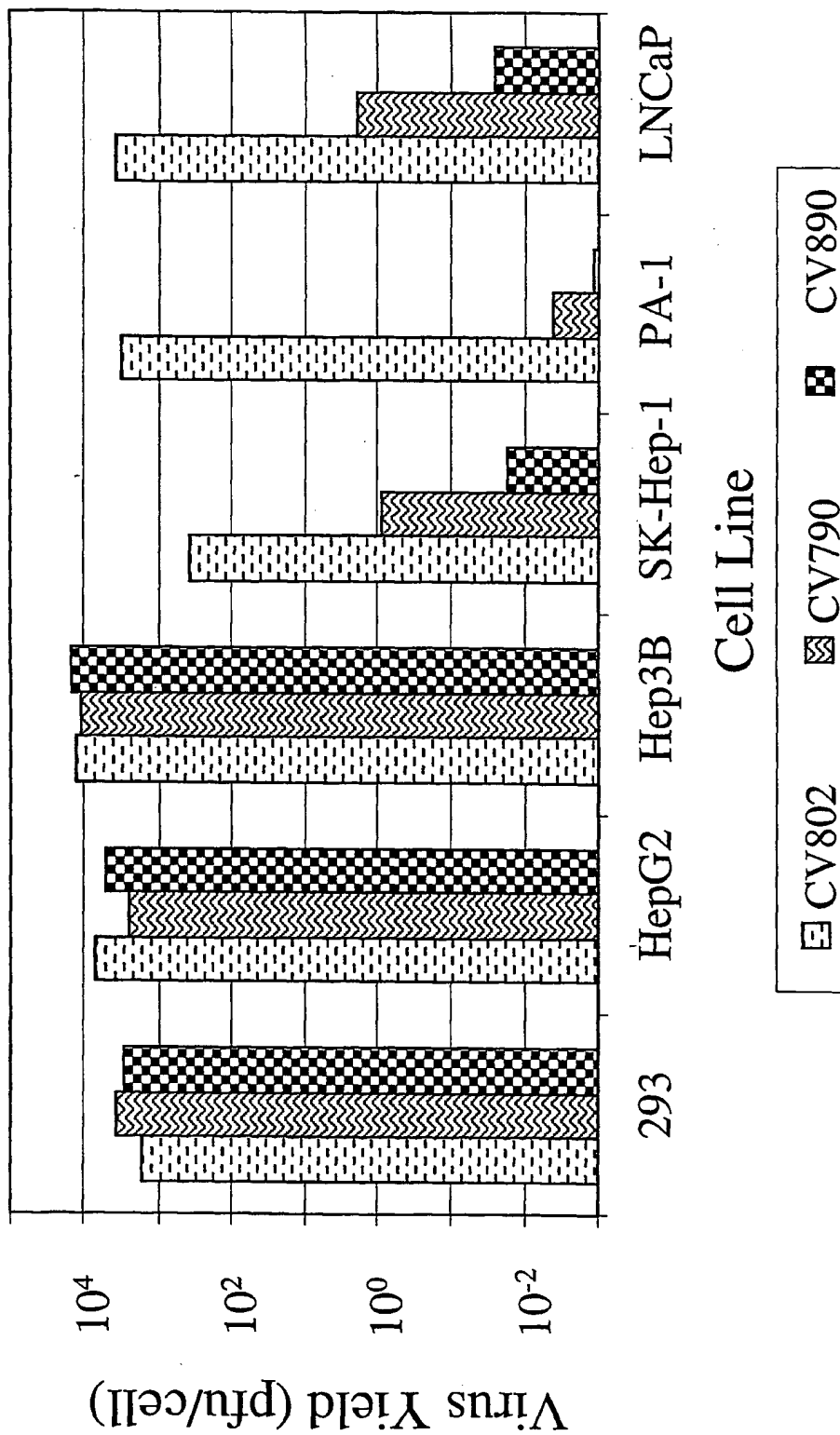
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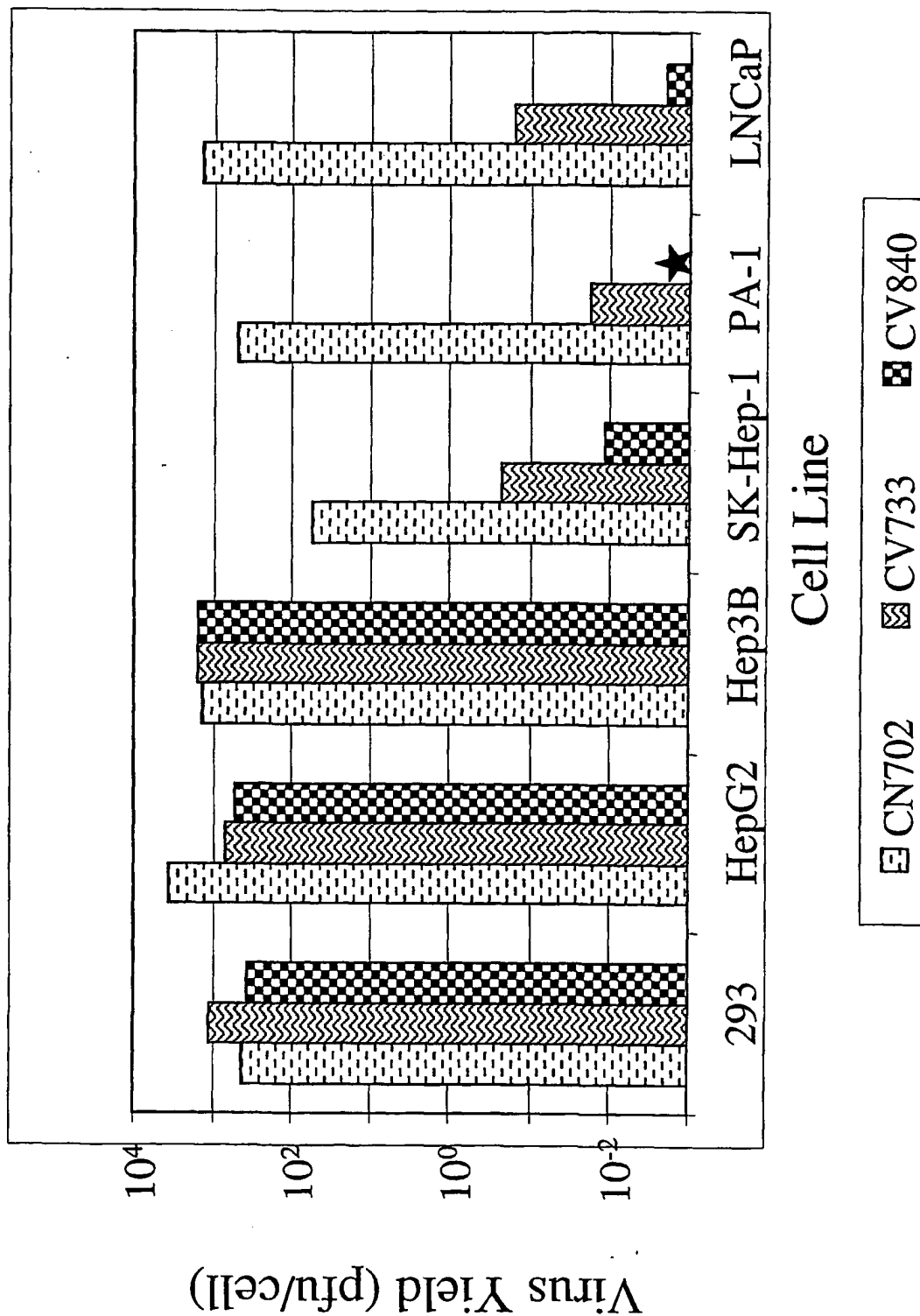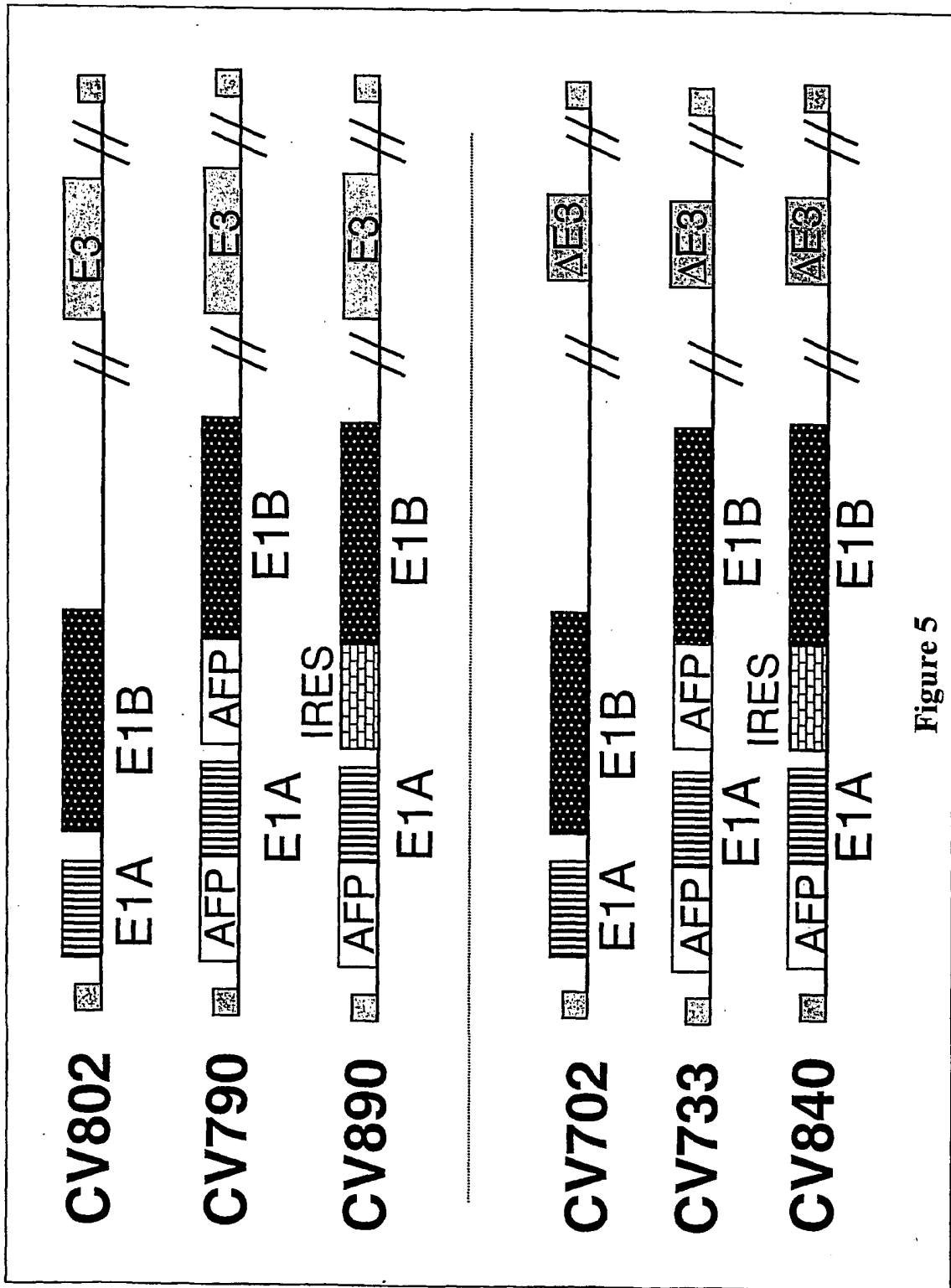
Figure 5

Region E3 of Adenovirus

Figure 6

Figure 7

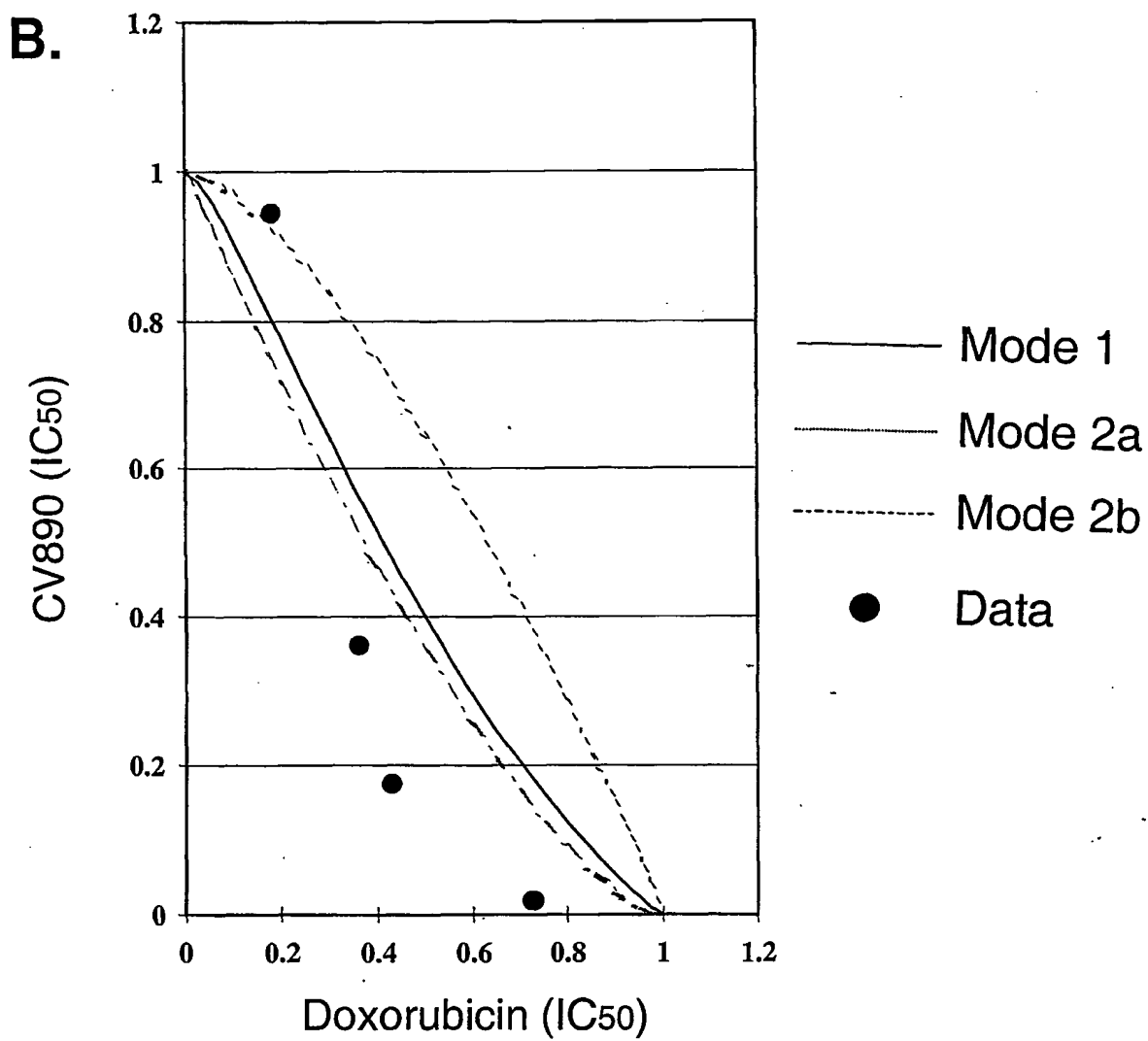Figure 8

FIG. 9

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      1                5                10                    15

ACT GCT ACC GGA CTA ACA TCT GCC CTA AAT TTA CCC CAA GTT CAT GCC
94
Thr Ala Thr Gly Leu Thr. Ser Ala Leu Asn Leu Pro Gln Val His Ala
                 20              25                    30

TTT GTC AAT GAC TGG GCG AGC TTG GAC ATG TGG TGG TTT TCC ATA GCG
142
Phe Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala
                 35                  40                  45

CTT ATG TTT GTT TGC CTT ATT ATT ATG TGG CTT ATT TGT TGC CTA AAG
190
Leu Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys
              50                  55                  60

CGC AGA CGC GCC AGA CCC CCC ATC TAT AGG CCT ATC ATT GTG CTC AAC
238
Arg Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn
          65              70                  75

CCA CAC AAT GAA AAA ATT CAT AGA TTG GAC GGT CTG AAA CCA TGT TCT
286
Pro His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser
    80                  85              90                  95

CTT CTT TTA CAG TAT GAT TAA
307
Leu Leu Leu Gln Tyr Asp
                 100
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Figure 10

Figure 11

Figure 12

Virus Yield of CV884

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